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MAGNETIC FIELD AND TISSUE ENGINEERING: IMPACT AND EFFECT ON CELL-MATERIAL INTERACTIONS.

TUTOR

Prof. Vincenzo D'Antò

Prof. Teresa Russo

PH.D. STUDENT

Dr. Valentina Peluso

A chi ha saputo sostenermi in questo lungo cammino.

A chi mi ama incondizionatamente.

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Chapter 1 Tissue engineering and medicine regenerative

Tissue engineering is an interdisciplinary field of research that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain or improve tissue function or a whole organ [1]. The use of tissue organ transplantation has been limited by donor shortage. Although the current use of drug therapy, medical devices and surgical reconstruction are generally available, many limits are related to the possibility of replacing the functions of the damage tissues or lost organs. In the case of traumatic bone loss, primary tumor resection and pathological fractures, bone is unable to heal itself. Clinical approaches involve bone grafts alone or in combination with other materials. If small bone defects are considered, autografts represent the gold standard as there is no risk of disease transmission or immune system rejection. Allografts could be considered as an alternative to reduce site morbidity and pain generally related to autografts. Tissue engineering and strategies based on the combination of scaffolds, cells and biomolecules are gaining an increasing importance as an alternative method in clinical therapy. Tissue engineering technologies lead to the development of 3D pore networks capable to fill the tissue void, provide structural support and deliver growth factors and/or cells that have the ability to form new tissues. Regenerative medicine strategies require interaction and integration with tissue and cells through incorporation of appropriate physical and cellular signals. Therefore, inclusion of modifying factors such as biologically active proteins and DNA are primary. Although basic functional tissue engineered strategies have been key there is still considerable scope for future developments of cell sources, individually tailored cell supports, immune modulation, vascularization, and the predictive abilities of computer and mathematical modelling for more complex materials.

1.1. Tissue engineering strategies

Two main approaches are utilized to produce a new engineered tissue. First, scaffolding can be used as a cell support device upon which cells are seeded in vitro; cells are then encouraged to lay down matrix to produce the foundations of a tissue for transplantation. The second approach involves using the scaffold as a growth factor or drug delivery device. This strategy involves the scaffold being combined with growth factors that recruited cells from the body to the scaffold site and form tissue upon and throughout the matrices. These two approaches are not mutually exclusive and can be easily combined. How a specific cell type and scaffolding are combined should be carefully matched to the specific new tissue to regenerate as it has been demonstrated that composition, topography and architecture of scaffolds are able to interact and influence cell behaviour. Scaffold architecture has been shown to modify the response of cells and subsequent tissue formation. [2] Nano to microscale topography has been demonstrated to affect cell behaviour by modification of cytoskeleton arrangements. [3] Furthermore, different cell types react to different materials. The source of cells is also an important choice for scaffolds, as is the cell culture methods used. [4]

1.2 Cells for tissue engineering

The production of an engineered tissue in vitro requires the use of cells to populate matrices and produce matrix resembling that of the native tissue. The main successes in this field have come from the use of primary cells, taken from the patient, and used in conjunction with scaffolds to produce tissue for re-implantation. However, this strategy has limitations, because of the invasive nature of cell collection and the potential for cells to be in a diseased state. Therefore, attention has become focused upon the use of stem cells, including embryonic stem (ES) cells, bone marrow mesenchymal stem cells (BM-MSCs) and umbilical cord-derived mesenchymal stem cells (UC-MSCs), periodontal ligament stem cells (PDLSCs).

1.2.1 Embryonic stem cells

ES cells could allow the production of type-matched tissues for each patient, either through stem cell banking or by the use of therapeutic cloning. ES cells have the ability to be maintained for long culture periods, therefore potentially providing large amounts of cells for tissues that could not be derived directly from a tissue source. Proof of the true pluripotent nature of ES cells is teratoma formation. This property demonstrates the ability of stem cells to tissue-engineer multiple tissue types but also highlights the importance of using a terminally differentiated cell stock without latent stem cell-like properties. The use of stem cells will therefore require a method to ensure differentiation by *in vivo* demonstration of an absence of teratoma formation. One of the critical steps of stem cell usage for regenerative medicine is therefore the ability to control the differentiation of the cells to the desired tissue lineages. Differentiation of ES cells has been achieved using protocols modified from BM-MSC protocols whereby ES cells can be directed to express features of bone, notably the accumulation of mineral. [5,6,7] However, there are indistinct steps in the use and differentiation of ES cells, notably embryoid body formation, which aids in the formation of ectodermal, endodermal and mesodermal lineages before terminal differentiation is initiated. Efforts have been made to understand this process but also to control the procedure using chemical aggregation. This process utilizes the affinity of Biotin to Avidin to provide a standardized and enriched system for differentiation. [8] This process has also been standardized by using cell suspensions, from which the cells are forced together using centrifugation. [9] Many stem cell lines are cultured on feeder cells to provide a conducive environment for growth, but there are implications for the transmission of xenogenic materials so systems for growing stem cells in feeder-free systems are being established. [10]

1.2.2 Bone marrow-derived mesenchymal stem cells

A stem cell type for bone and cartilage repair is the adult BM-MSC; these cells have been shown to be able to differentiate from a generic marrow cell population to an osteogenic lineage and have been used to augment repair of bone. [11,12,13] The MSC cell population can be isolated as a fraction of the adherent bone marrow colony forming units – fibroblastic (CFU-F) [14] and can be differentiated to the osteogenic and other lineages. [15,16] As marrow is a complex mixture of cells a more defined starting cell population subset can be isolated from the mixture on the basis of epitope expression such as Endoglin [17,18] and STRO-1. [19,20] These cells

can be removed from marrow and used to enhance materials such as the filler used for stabilizing artificial hip joints or for joining critical sized defects in bone. [21]

1.2.3 Cord derived mesenchymal stem cells

Since the discovery that umbilical cord blood contains MSCs that can undergo multi-lineage differentiation, much research has been focused on determining their applications. The analysis of their gene expression profile reveals similarities to BM-MSCs [22], with an ability to differentiate into adipocytes, osteoblasts [23], hepatocytes [24] and neuronal-like cells. [25] This type of stem cell does function as a BM-MSC and are capable greatly improve the availability of matched tissues for treatments. This source of stem cells provide a large pool of material, which can be purified using non-invasive techniques and can be recipient matched. In addition, as BM-MSCs differentiating potential may decrease with age [26,27] an alternative such as UC-MSCs can be of huge benefit.

1.2.4 Periodontal ligament stem cells

The discovery of periodontal ligament stem cells (PDLSCs) highlighted the possibility for development of tissue engineering technology-based therapeutics for disrupted periodontium. PDLSCs are a kind of somatic stem cells that show potential to differentiate into multiple cell types and undergo robust clonal self-renewal. Therefore, PDLSCs are considered a highly promising stem cell population for regenerative therapy in periodontium; however, their rarity prevents the progression of basic and clinical researches. PDLSCs obtained from mature periodontal ligaments possess stem cell properties similar to MSCs rather than neural crest cells.[28,29] More specifically, PDLSCs express MSC surface markers (CD105 [30-34], CD90 [30,35], and CD73 [36]) but lack expression of CD45, CD34, and CD14 or CD11b, CD79a, or CD19 and HLA class II [34]. Moreover, PDLSCs located in the perivascular wall of periodontal ligaments share similarities with pericytes in morphology, differentiation potential, cell phenotype (expression of pericyte-associated markers CD146, neural/glial antigen-2 and CD140B), and the ability to form capillary-like structures in vitro. [37]. Recent studies show that MSCs obtained from various tissue types (brain, lung, liver, kidney, muscle, skin, and bone marrow) also exhibit pericyte characteristics [38, 39]. In addition, PDLSCs have an immunomodulatory ability that is comparable to bone marrow MSCs [32]. First, PDLSCs possessed low immunogenicity due to the absence of HLAII DR or T cell costimulatory molecules (CD80 and CD86) [40].

Secondly, PDLSCs inhibited proliferation of allogeneic T cells through upregulation of cyclooxygenase-2 (COX2) and prostaglandin E2 (PGE2) [41]. Surprisingly, after osteogenic induction, the inhibitory effect of PDLSCs on T cell proliferation was intact [42]. Third, PDLSCs suppressed B cells proliferation, differentiation, and migration through cell-to-cell contact, which was mediated by programmed cell death protein-1 [43]. The low immunogenicity and immunosuppressive effects on T and B cells allow use of allogeneic PDLSCs in periodontal regeneration. Indeed, allogeneic PDLSCs have been tested in a sheep [44] and a swine model, and the therapeutic effects of allogeneic PDLSCs were equal to those of autologous

PDLSCs. In summary, PDLSCs are a subpopulation of MSCs located in the perivascular space which share similarities with pericytes. However, there is no standard criterion for the identification of PDLSCs.

1.3 Scaffold characteristics

As the successful tissue engineering progresses, the need for novel scaffold structures and techniques has become of essential importance. The manner and the process in which biodegradable polymers, such as the common poly lactic acid (PLA), are become innovative allowing the final properties of the scaffold to be tailored. Nowadays, we can have high-pressure CO_2 foamed scaffolds, injectable scaffolds, custom scaffolds, further modified using growth factors, zonation of materials and plasma polymerization deposition. For many years, poly-hydroxyl acids such as PLA and poly lacticoglycolic acid (PLGA) have been used for tissue engineering procedures, as they have a simple degradation by hydrolysis, providing a determinate drug release and a degradation related with tissue in growth. With particular attention to molecular weights, side chains and cross links, materials can be performed with tailor made properties. Moreover, poly-hydroxyl acid materials also are used *in vivo* usage as degradable sutures, biodegradable surgical components and drug delivery devices. Scaffolds are materials engineered to cause favourable cell -material interactions to contribute to the formation of new functional tissues. Scaffolds mimic the extracellular matrix of the native tissue allowing cells to influence their own microenvironments. The scaffolds should allow cell attachment and migration, deliver cells and biochemical factors, enable the diffusion of vital cell nutrients, exert certain mechanical and biological influences to modify the behaviour of cell cycle phases. A concrete example is when in 2009, Thorsten Walles and his interdisciplinary team of thoracic surgery implanted the first bioartificial transplant with an innate vascular network for post-transplant graft supply successfully into a patient awaiting tracheal reconstruction. [45] To achieve the goal of tissue reconstruction, scaffolds must have some specific requirements; high porosity and adequate pore size are necessary to facilitate cell seeding and diffusion of cells and nutrients into the scaffold. Scaffold must be biodegradable preferably to be absorbed by the surrounding tissues without the necessity of surgical approach removal. The degradation rate has to coincide as much as possible with the rate of new tissue formation. Scaffolds should be both solid and Injectable based on the clinical uses. Finally, is crucial a good control of the 3D environment to ensure reproducibility of experiments and offer better results.

1.4 Materials

In the tissue engineering field, many different materials have been investigated : natural and synthetic, biodegradable and permanent.[46] Most of these materials have been known in the medical field before the advent of tissue engineering , being already employed as bioresorbable sutures, such as collagen and some polyesters. In the last years, new biomaterials have been engineered to have ideal properties and different functional customization: injectability, biocompatibility, synthetic manufacture, transparency, nanoscale fibers, low concentration, non-immunogenicity, resorption rates, etc. A commonly used synthetic material is PLA - polylactic acid. This is a polyester which degrades within the human body to form lactic acid, a naturally

chemical. Similar materials are polyglycolic acid (PGA) and PCL: their degradation mechanism is similar to that of PLA, but they show different degradation rates, respectively a faster and a slower rate of degradation compared to PLA. The disadvantage is that these materials have a hydrophobic nature, that could inhibit their biocompatibility. [47] In order to fix the lack of biocompatibility, much research has been done to combine these hydrophobic materials but more structural integrity with hydrophilic and more biocompatible hydrogels. By combining the two different types of materials produces a more biocompatible tissue scaffold. [48] Scaffolds may also be constructed from natural materials: different derivatives of the extracellular matrix have been studied to evaluate their ability to support cell growth. Protein materials, such as collagen or fibrin, and polysaccharidic materials, like chitosan [49] or glycosaminoglycans (GAGs), have all proved suitable in terms of cell compatibility, but could have a potential immunogenicity. Among GAGs there is hyaluronic acid, possibly in combination with cross linking agents (e.g. glutaraldehyde, water-soluble carbodiimide, etc.). Another form of scaffold is decellularised tissue extracts whereby the remaining cellular remnants/extracellular matrices act as the scaffold. Recently, nanocomposites biomaterials were obtained by incorporating nanomaterials within the polymeric matrix to carried out bioactive scaffolds.[50] Moreover, it is possible to manipulate gradients of soluble molecules, and to characterize cells in these complex gradients more effectively than conventional 3D cultures based on hydrogels, cell spheroids, or 3D perfusion reactors.[51]

1.5 Synthesis

Several different methods have been described in the literature for preparing porous structures to be employed as tissue engineering scaffolds. Each of these techniques presents its own advantages and disadvantages; some are described below. Nanofiber self-assembly: one of few methods for creating biomaterials with properties similar in scale and chemistry to that of the natural *in vivo* extracellular matrix (ECM). [52] Textile technologies: techniques for the preparation of non-woven meshes of different polymers. In particular, non-woven polyglycolide structures have been tested for tissue engineering applications: such fibrous structures have been found useful to grow different types of cells but is difficult obtaining high porosity and regular pore size. Solvent casting and particulate leaching (SCPL): ideal for the preparation of structures with regular porosity, but with limited thickness. First, the polymer is dissolved into an organic solvent, then the solution is cast into a mold filled with porogen particles such as sodium chloride, crystals of saccharose, gelatin spheres or paraffin spheres. The size of the porogen particles will affect the size of the scaffold pores, while the polymer to porogen ratio is directly correlated to the amount of porosity of the final structure. After the polymer solution has been cast the solvent is allowed to fully evaporate, then the composite structure in the mold is immersed in a liquid for dissolving the porogen: water in the case of sodium chloride, saccharose and gelatin or an aliphatic solvent like hexane for use with paraffin. Once the porogen has been fully dissolved, a porous structure is obtained. The organic solvents which must be fully removed to avoid any possible damage to the cells seeded on the scaffold. Gas foaming: this technique using gas as a porogen. First, disc-shaped structures made of the desired polymer are prepared. The discs are then placed in a chamber where they are exposed to high pressure CO_2 for several days. The pressure inside the chamber is gradually restored to atmospheric levels

and the pores are formed by the carbon dioxide resulting in a sponge-like structure. Emulsification freeze-drying: a synthetic polymer is dissolved into a solvent (e.g. polylactic acid in dichloromethane) then water is added to the polymeric solution and the two liquids are mixed in order to obtain an emulsion. Before the two phases can separate, the emulsion is cast into a mold and quickly frozen in liquid nitrogen. The frozen emulsion is subsequently freeze-dried leaving a solidified porous polymeric structure. Freeze-drying by itself is also a commonly employed technique for the fabrication of scaffolds. Thermally induced phase separation TIPS: requires the use of a solvent with a low melting point that is easy to sublime. For example, dioxane could be used to dissolve polylactic acid. Phase separation is induced through a small quantity of water. Following cooling below the solvent melting point and some days of vacuum-drying to sublime the solvent, a porous scaffold is obtained. Electrospinning: technique that produce continuous fibers from submicrometer to nanometer diameters. the solution is fed through a spinneret and a high voltage is applied to the tip. The buildup of electrostatic repulsion within the charged solution, causes it to eject a thin fibrous stream. A mounted collector plate or rod with an opposite or grounded charge draws in the continuous fibers, which arrive to form a highly porous network. By modifying variables such as the distance to collector, magnitude of applied voltage, or solution flow rate is possibly change the scaffold architecture. CAD/CAM technologies: computer assisted design and manufacturing techniques. A 3D structure is designed using CAD software. The porosity can be tailored using algorithms within the software. The scaffold is then realized by using ink-jet printing of polymer powders or through Fused Deposition Modeling of a polymer melt.[53] Laser-assisted bioprinting: technique arranges of small volumes of living cell suspensions in set high-resolution patterns.[54] Additive manufacturing: an innovative method of construction uses an ink-jet mechanism to print precise layers of cells in a matrix of thermoreversible gel. The field of three-dimensional and highly accurate models of biological systems is pioneered by multiple projects and technologies including a rapid method for creating tissues and even whole organs involve a 3D printer that can print the scaffolding and cells layer by layer into a working tissue sample or organ. The achieved biocompatibility was comparable to the commercially available collagen membranes. Injectable materials for tissue engineering: a novel biodegradable, particulate, scaffold system which can be injected into a site of bone trauma. [55] The dynamics of this scaffold type allows injection at room temperature and solidification at body temperature allowing for a non-invasive delivery system for treatment of non-union bone defects. Microparticles are small enough to be delivered by syringe and can be used as an injectable scaffold.

1.6 Bone regeneration

The tissue engineering principles can be applied to each type of tissue to be regenerated, hard or soft tissue. During these years of research my goal has been the study of the mechanisms underlying bone tissue regeneration. For this reason, a small introduction on osteogenesis is important.

Bone tissue is part, together with cartilage, of specialized connective tissues with a supporting function. The belonging of bone tissue to connective tissues is justified both by its origin from the mesenchyme and by its constitution, being formed by cells and intercellular substance composed of collagen fibers. The peculiarity of

the bone tissue is that it is mineralized: in fact, the intercellular substance is for the most part impregnated with mineral crystals, mainly calcium phosphate. Its components give this fabric outstanding mechanical properties of hardness and resistance to pressure, traction and torsion. In addition, given the high content of calcium salts, bone tissue represents the main deposit of calcium ion for the metabolic needs of the whole organism. From a macroscopic point of view, there are two varieties of bone: cancellous bone and compact bone. Spongy bone is found mainly in the short bones, flat bones and epiphyses of long bones, shaped like a sponge, with variously oriented and intersecting trabeculae and delimiting marrow cavities filled with hematopoietic bone marrow. The compact bone is found forming the most superficial portion of the short bones, flat bones and long bones, as well as forming the diaphysis of the latter. It is devoid of macroscopically evident cavities. the bone tissue contains a significant amount of organic intercellular substance, composed of connective fibers and fundamental substance.

The mineral component is also added to the organic component. The connective fibers are represented almost entirely by collagen fibers, composed of type I collagen. The collagen microfibrils have the typical transverse striation with a period of 70 nm. They aggregate to form collagen fibers of considerable thickness (5-10 μm) only in the so-called fibrous bone tissue, while in the lamellar tissue, the microfibrils of collagen (about 60 nm thick) does not join into fibrils but form a homogeneous felt. From the layer of connective tissue that externally envelops the bone, said periosteum, thick bundles of collagen fibers branch off that penetrate inside the cortical bone tissue and are lost in the intercellular substance of the bone: these bundles constitute Sharpey's perforating fibers, which anchor the periosteum to the surface of the bone. The fundamental substance has a peculiar composition and to a good extent different from that of the other connective tissues. It includes various classes of macromolecules: Proteoglycans, composed of acidic glycosaminoglycans, usually sulphured, joined by short protein chains. Among these we find: Proteoglycan type I (PG-I), also called biglycan as it consists of two molecules of chondroitin sulfate joined at one end by a polypeptide rich in leucine.

Proteoglycan type II (PG-II), also called decorin because it is associated with the collagen microfibrils as to decorate them. It consists of a protein part similar to that of PG-I combined with a single molecule of chondroitin sulfate. It is hypothesized that it has a role in guiding the deposition of mineral crystals along the collagen microfibrils.

Glycoproteins, usually phosphorylated or sulphated, include different molecules some of which are believed to play a fundamental role in the control of mineralization. These include: Osteonectin, the most abundant glycoprotein. It has a high affinity for calcium. It is believed to act as an element of nucleation of mineral crystals, as it is considered capable of concentrating calcium in its vicinity thus creating the conditions to start the precipitation of calcium phosphate. Alkaline phosphatase, an enzyme capable of hydrolyzing phosphate groups bound to organic substrates active in an alkaline environment (pH 8-10). It could play a role in mineralization processes, making phosphate ions available for the formation of mineral crystals. According to others, it is instead involved in the synthesis of the organic bone matrix. Fibronectin, an adhesion molecule located mainly in the pericellular matrix and characterized by a portion capable of binding to collagen.

Sialoproteins, BSPs, peculiar glycoproteins containing glycidic residues of sialic acid. These proteins possess a particular amino acid sequence Arg-Gly Asp (RGD sequence) which in in vitro experiments has been seen to mediate adhesion to the substrate of various cell types, including bone cells. It is therefore believed that bone sialoproteins have the physiological function of allowing cells to adhere to the bone matrix.

Proteins containing γ-carboxyglutamic acid (GLA), a particular amino acid derived from glutamic acid with an additional carboxylic group linked to carbon in the γ position. Bone proteins containing GLA are of two types: Osteocalcin, or bone GLA protein, a small protein containing 3-5 GLA residues. It has been hypothesized that it may play a role in inhibiting mineralization as it is believed to be able to bind to the calcium ion and make it unavailable for combination with the phosphate ion, thus inhibiting the dimensional growth of mineral crystals. Osteocalcin abounds in mature bone tissue and is scarce in bone in the process of formation; GLA matrix protein, with a molecular weight greater than osteocalcin, is present in both mature and developing bone, as well as in cartilage destined to be replaced by bone tissue, such as growth cartilage.

The mineral component is represented by crystals of calcium salts, mainly calcium phosphate to which smaller quantities of calcium carbonate and traces of other salts are added (calcium fluoride, magnesium phosphate). Calcium phosphate is present in the form of apatite crystals, whose unit cell has the shape of a flattened hexagonal prism; the two positive charges of the chemical structure are normally neutralized by the bond with two hydroxyl ions (OH-), thus forming hydroxyapatite.

1.6.1 Bone tissue cells

The cells of the bone tissue are morphologically distinguishable in 4 varieties: osteoprogenitor cells (also called preosteoblasts), osteoblasts, osteocytes and osteoclasts. Of these, osteoprogenitor cells, osteoblasts and osteocytes are consecutive functional phases of the same cell type, derived from the osteogenic differentiation of the pluripotent mesenchymal cell of the connective tissues. Osteoclasts, on the other hand, derive from precursors immigrated into the bone tissue from the blood, called preosteoclasts, which differentiate from stem cells of the hematopoietic bone marrow. Osteoprogenitor cells, or preosteoblasts, have a spindle or oval shape, with sparse and basophilic cytoplasm and euchromatic nucleus with large nucleolus. Osteoprogenitor cells have a proliferative capacity, which manifests itself in a particular way during body growth. They are able to produce and secrete bone morphogenetic proteins (BMPs), autocrine growth and differentiation factors. When they enter the path of differentiation, the osteoprogenitor cells transform into osteoblasts. Osteoblasts are the cells responsible for the synthesis of the intercellular substance of the bone and its mineralization. They have a globular or polyhedral shape and tend to form epithelioid laminae close to the bone surfaces being formed. Osteoblasts have significant dimensions (about 20 μm), a roundish, euchromatic nucleus, with a large nucleolus and an abundant and basophilic cytoplasm in which PAS-positive granulations can be highlighted. Osteoblasts are joined and with neighboring osteocytes via tight junctions (or gap junctions), through which cells exchange signal molecules for the coordination of metabolic activity and deposition of the bone matrix. The osteoblast is the site of synthesis of the organic molecules of the intercellular substance of the bone.

osteocytes are the typical cells of mature bone, responsible for its maintenance and also capable of initiating its remodeling. They are terminal cells, with a finite autonomy of life, finely regulated by endocrine mechanisms. The osteocyte is a star cell, with a cell body in the shape of a biconvex lens and numerous cytoplasmic extensions. Osteoclasts are the cells responsible for bone resorption. The precursors of osteoclasts, called preosteoclasts, originate in the hematopoietic bone marrow and are related to the differentiation line of a category of white blood cells, the monocytes.

Preosteoclasts are transported by the circulatory stream to the sites where bone resorption processes must take place; arrived here, they migrate into the bone tissue and fuse together giving rise to active osteoclasts, syncytial elements capable of dissolving the mineral component and enzymatically digesting the organic components of the bone tissue. Mature osteoclasts are giant cells (100-200 μm), plurinucleated as they originate from the fusion of single mononuclear precursors: in a single osteoclast they can in fact be present up to 50 nuclei, with lax chromatin and clearly evident nucleolus. The cytoplasm is acidophilic. Osteoclastic function is finely regulated by hormonal and local factors. Osteoclasts are the only bone cells that possess receptors for the hormone calcitonin, produced by the parafollicular cells (or C cells) of the thyroid, with action antagonist to parathyroid hormone.

1.6.2 Osteogenesis

The process of organogenesis of bone tissue follows a general pattern in which pluripotent cells receive signals that prompt them to participate in a development program and in the specification of a particular cell lineage. The ontogenesis of the skeleton is no exception: in fact, the mesenchymal cells must receive information signals that enable them to form bone and cartilage. Often these signals come from the surrounding tissues; in some regions signals starting from the cartilage induce the formation of bone in adjacent areas while in other regions the signal that osteogenesis begins comes from the epithelium. [56] Before the start of the osteogenic process, the mesenchymal cells are morphologically homogeneous and appear flat and shaped like fibroblasts. They also have a large contact surface with adjacent cells with which they communicate through gap junctions. The first step of osteogenesis is the condensation process, in which the mesenchymal cells aggregate, transforming into an epithelium whose shape is predictive of the future bone element. During this transformation, the cells increase in density and lose their fibroblastic appearance, becoming rounded. They also begin to express various adhesion molecules, such as N-cadherin, fibronectin and N-CAM (Neural Cell Adhesion Molecule). Their expression is then decreased as cells begin to express differentiation markers osteoblastic. [57] In fact, multipotent MSCs undergo asymmetric divisions that generate osteoprecursors, preosteoblasts, functional osteoblasts and finally osteocytes. This progression from one differentiation stage to another is accompanied by the activation and inactivation of various transcription factors such as Cbfa1 / Runx2, Msx2, Dlx5, Osx and by the expression of later functional markers such as Osteopontin, Type I Collagen, Alkaline Phosphatase, Bone Sialoprotein, Osteocalcin. [58]

1.7 Tissue engineering in dentistry

The traditional techniques based on restored missing or deteriorated tissue with autologous grafts from living donors are still used in dentistry as well as in other medical fields, despite their disadvantages, such as risk of infections and rejection post transplantation. An innovative alternative is provided by regenerative medicine, that is able to regenerate, repair, or replace tissues and to ensure restoration of their function by combining tissue engineering with the self-healing ability of tissue. In vitro tissues engineering involves the emerging field of biotechnology in a multidisciplinary approach with different topics such as medicine, materials science, cell and molecular biology, bioengineering, and genetics [59]. This approach is being increasingly used in dental and maxillofacial reconstruction medicine, providing a new option for the reconstruction of teeth, periodontium, bones, oral mucosa, conjunctiva, skin, temporomandibular joint, both bone and cartilage as well as nerves, muscles, tendons, and blood vessels of the oral and maxillofacial area [60]. Defects in the oral area represent the main targets of soft or hard tissue regeneration: maxillofacial defects, periodontal diseases (gingiva inflammation, PDL, alveolar bone, and cementum loss), dental pulpal diseases, and hard tissue defects of the tooth [61].The fields of application are several, below is a brief review of the main ones.

Regenerative endodontic therapy (RET) is defined as "biologically based procedures designed to replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp–dentin complex" [62]. Regenerative endodontics aims to restore normal function of the pulp, by regenerating the dentin–pulp complex damaged by infection, trauma, or developmental anomalies of immature permanent teeth with necrotic pulp. The benefits of regenerative endodontics not only stand in revitalization of the tooth, but also continued root development and, potentially, increasing fracture resistance [63].

Regenerative Periodontics: considered a distinctive tissue structure, periodontal tissue consists of a three-dimensional complex of alveolar bone, PDL, and cementum. The incidence of periodontal disease, the main cause of tooth loss, is increasing among the population. It has a microbial cause and, in most cases, results in irreversible destructive phenomena. Chronic inflammation severely affects the periodontium, leading to the resorption of the alveolar bone, a pathological phenomenon that cannot be stopped by natural processes [64,65]. Nonsurgical periodontal therapies, such as scaling and root planning, represent the first choice methods in preventing disease progression in its first stages, but the removal of pathogens and necrotic tissues provide only partial, local regeneration of the periodontal tissue. Surgery, needed in the advanced stages, or other currently common periodontal therapies, such as growth factors and grafts, could be replaced by the use of stem cells therapy as a successful method for treating periodontal diseases due to the existence of stem cells in the PDL [66,67]. The target of regenerative dentistry is to develop effective therapies and techniques to treat periodontal diseases using applied tissue engineering and regeneration on the lost or affected support tissue of the periodontium: alveolar bone, periodontal ligament, and cementum. Two major strategies for periodontal regeneration have been outlined: guided tissue regeneration (GTR) and tissue engineering .GTR, a regenerative surgical technique that aims to prevent apical migration of the epithelium in the bone defect by placing a membrane at the root surface [68,69]. The second choice is tissue engineering strategy that may take two

approaches, scaffold-free or scaffold-based [70]. The scaffold-free approach uses cells or cell aggregates transplanted onto the wound area with no carrier cell. Clinical studies reported that PDLSCs and DPSCs have the potential to form periodontal tissues, but problems occur with cell diffusion out of the defect zone. The better alternative is the scaffold-based approach [71]. Multiphasic scaffolds, with distinctive particularities of each layer both in architecture and chemical/biochemical composition, are required to imitate the complex structure of the periodontium. Additionally, 3D substructure contributes for cell adhesion and movement, and contains growth factors facilitating the signal delivery needed for morphogenesis and differentiation. Biomaterials and controlled drug delivery for periodontal regeneration involve the use of inorganic, polymeric, or composite biomaterials. For bone and cementum repair/regeneration, inorganic biomaterials are the material of choice due to their similarities in composition and mechanical properties. For PDL regeneration, polymeric biomaterials are appropriate. By combining inorganic and polymeric biomaterials, biomimetic scaffolds for bone and cementum regeneration can be fabricated [72]. Many studies with the objective of regenerating the periodontal tissues highlighted that the actual biomaterials cannot exactly mimic the natural architecture of periodontal tissues, so the connections between their components, cementum–PDL–alveolar bone, remain unstable and cannot support teeth or bear occlusal force [73]. Thus, bioinspired innovative materials, with shape and molecular composition at a manometer scale controlled, are needed to mimic the complex structure of periodontal tissues at the micro- and nano- level.

Regenerative Oral and Maxillofacial Surgery: Oral and maxillofacial surgeries play important roles in the treatment of traumatic and degenerative disease with tissue loss. The techniques used have been improved over time, from using growth factors and platelet concentrates to biomaterial scaffolds, autologous tissues and stem cells. In this field, tissue engineering and regeneration are the approaches currently available for achieving the goals of reconstruction procedures [74]. In maxillofacial reconstruction, surgeons have two main objectives: to provide the anatomic form and the function of the oral-maxillofacial area. Due to the facial skeleton has a complex structure, reconstruction is complicated because it must restore the volume, shape, bone continuity, and symmetry of the skeletal bone. Moreover, the numerous soft and hard tissues that form this area provide important functions such as articulation, facial expressions, mastication, swallowing, and breathing, thus the reconstruction must restore, maintain, and stabilize these tissue functions. In addition, the reconstruction must be performed not only for reconstructive goals but also for aesthetic goals. [75].

Oral and maxillofacial surgery can use MSCs from the oral cavity, which are an important and easily accessible source to the surgeon. Several maxillofacial bone defects can be approached using bone tissue regeneration. Soft tissue, such as skin and oral mucosa, can also be regenerated. Cartilage regeneration, salivary gland regeneration, fat, muscle, blood vessels, and nerve regeneration represent other applications of tissue engineering in oral and maxillofacial surgery [60,73]. Recent studies highlight the possibility of using GMSCs as the cellular components for 3D bioprinting of scaffold-free nerve constructs needed for peripheral nerve repair and regeneration [76] or for treating gingival defects [77].

Bone regeneration: Substantial bone defects of the maxilla and mandible, in need of surgery, originate from congenital abnormalities, accidental traumatic injuries, tooth extraction, surgical resection of benign or malign tumors, and infections. The most challenging situation for the maxillofacial surgeon is the restoration of large bony defects due to trauma or post-resection. In the standard reconstruction of maxillofacial bone defects, autologous grafting is still the gold standard technique, even if it presents many disadvantages. A perfect technique and material for bone reconstruction has not yet been found, even if many clinical approaches have been attempted in recent years. Bone tissue engineering techniques provide a solution for reconstructing large size bone defects in the oral and maxillofacial region using autologous bone grafts, conditioned by adequate vascularization. MSCs are a key element in bone regeneration due to their capacity to induce bone regeneration by mimicking biological processes [78] and through the secretion of cytokines and growth factors. Several investigations in bone tissue engineering have reported various types of MSCs combined with different scaffolds as potentially suitable for regeneration for surgical procedures in the oral and maxillofacial region [79-82]. A clinical research study reported biocomplexes fabricated from DPSCs and collagen sponges in human mandible repair with remarkable results [83]. Finally, three-dimensional-engineered scaffolds complexed with gingiva-derived mesenchymal stem cells (GMSCs) could provide a new therapeutic approach to improving bone tissue regeneration [84].

Cartilage regeneration: The temporomandibular joint (TMJ) is affected by many diseases and defects that can compromise the cartilaginous layer of the condyle. Cartilage is an avascular tissue that has a limited capacity to repair because of limited supplies of nutrients. Many surgical procedures are available for TMJ disorders, but all are aggressive and dangerous for the patient [85]. A recent research objective is the insertion a cell source to manufacture neocartilage after displacement of the dysfunctional disc. Biocompatible scaffolds seeded with cells and biological modulators can be useful in such a process. Thus, the regeneration process of the TMJ is based on several main factors, such as scaffold design and material, stem cells, bioactive agents, biochemical compatibility between the scaffold and the surrounding environment, and the ability of the host to accept the scaffold and facilitate tissue formation. Collagen, gelatin, hyaluronic acid, fibrin, silk, agarose, polylactic acid, or poly vinyl alcohol are only some of the materials that can be used in cartilage tissue engineering [86]. Extracting stem cells from the synovial capsule surrounding the joint holds has been proven to be a promising choice for generating neocartilage [87].

Tooth regeneration: The regeneration of the entire tooth and its replacement represent the final objective of tooth tissue engineering. Even if dental tissues have no capacity for self-regeneration, the teeth are an important source of stem cells. This technique could be used to create replacements for dental implants and eliminate the risk of rejection [88]. Tooth regeneration research using adult stem cells has been considered. Autologous DPSCs or postnatal tooth germ cells have limited window of availability, so they can only provide a casual source for whole-tooth regeneration. The experiments on animals have shown that tooth-like organs, with dentin and enamel, can be developed by ectopic subcutaneous grafting these cell aggregates under the renal

capsule or into the anterior eye chamber [89]. After implantation into the animal's jaw, a whole tooth could be generated. Nevertheless, entire-tooth engineering or regeneration is still complicated.

During the last decade, tissue engineering based on stem or progenitor cells is become a promising approach for restoring the integrity of dental and maxillofacial tissues. Major progress has been achieved for the improvement of the properties of materials used in dentistry. It was shown how these materials are improving the treatments in mainly all the important areas of dentistry, such as endodontics, periodontics, implants, tissue engineering and restorative dentistry. Research and clinical applications of dental staminal cells have proven their utility and advantages, such as the capacity for self-regeneration and multidirectional differentiation, easy accessibility, and, importantly, low autologous transplant rejection. However, for real and stable tissue regeneration in dentistry many other theoretical and technological approaches must be applied in the future. Future studies are still necessary to identify suitable stem cells for performing the physiological role of native tissue, growth factors able to support both cellular differentiation and replication, and to determine the role of microvascularization in tissue regeneration. Regeneration of the entire tooth is a major objective for replacing classical dental implants and overcoming their disadvantages. Such an approach would allow the reconstruction or regeneration of teeth in the future, significantly increasing the quality of dental health.

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Chapter 2 Magnetic field and living organisms

Living organisms are complex electrochemical systems that evolve under different chemical and physical stimuli. They are able to adapt to external environment also through the influence of natural magnetic field. An example is our optical system; the eye responds to the influence of electromagnetic spectrum. Magnetic interactions are used by our body to regulate different cellular function such as circadian rhythms or neuronal activities. And this because biological macromolecules are subjected to strong magnetic consequences such as the protein that have their specifical folding and their intermolecular interaction thanks to specifical magnetic forces. Accordingly, multiple frequencies, modulations and magnetic wave intensities are perceived by living organisms, starting from the cells, and consequently provoke biological responses and effects. This point of view offers a completely different perspective from which to analyze the systems that allows to the integrate interpretations based on molecular biology and the biochemistry. Several approaches, already for many years, use electric and magnetic fields in clinic with good results, for example magnetotherapy in traumatology and orthopaedics [1,2] or transcranial magnetic stimulation for depression treatment. [3] The problem remains the lack of precise scientific knowledge that guides the choice of one radiation rather than another to interact with a specific biological mechanism. Therefore, the mechanism that allow a single cell to react to signals of such intensity that they cannot be theoretically coupled to any physiological event become difficult to explain. So, in this scenario, investigating the characteristics of a magnetic field and its interactions with biomaterials and different types of cell lines becomes interesting.

2.1 Electric fields, magnetic fields, electromagnetic fields

The electric field is the physical size that characterizes a region of space whose properties depend on the distribution of electric charges. It is manifested by a force acting on any electric charge introduced into the electric field seat space; it is described by a **vector E** (called electric field vector, or simply electric field) which at any point in the space region indicates the direction, the intensity and the direction of the force acting on a single point charge placed at that point; its intensity is measured in volts per metre (V/m); because of the force exerted on the charges, the electric field is capable of generating electric currents in so-called conductive materials. The magnetic field is the physical size that characterizes a region of space whose properties depend on the distribution of electric currents. In particular, a magnetic field is manifested by a force acting on any other electric current or charge electric not at rest introduced into space magnetic field seat; can be described by an H vector (called magnetic flux density, or also magnetic induction) defined by the force that at each point in the region of space manifests itself on an elementary current or electric charge not at rest, placed at that point; its intensity is measured in **tesla (T).**

The effects of the interaction of a charged particle with a magnetic field are described by the force of Lorentz. This force originates from the magnetic field, and acts on a charge particle q entering with velocity v into a magnetic field B, and is represented from the vector product: **F=qv B**. If the vector v is parallel to the magnetic field vector B, then the force module is equal to zero and on the charged particle does not act any force that disturbs its motion. If instead a charge enters with a velocity perpendicular to the field, this will move by uniform circular motion. Finally, if the

velocity vector is oblique with respect to the field vector magnetic motion will result in a spiral or helical motion of the particle. [4]

Electric charge and electric current are therefore, as previously mentioned, the material sources of the electric field and magnetic field respectively. An electric field can be generated, in addition to a distribution of electric charge, even by a magnetic field that varies in time. Similarly, a magnetic field can be generated not only by a distribution of electricity, but also by a field electric variable in time. So, with time as variable, electric field and magnetic field become one the source of the other: in these conditions, electric field and magnetic field can be considered as two aspects of a single physical size, the electromagnetic field, capable of propagating to an indefinite distance from the source. [5] In specific spatial conditions, source geometry and wavelengths, the amplitude of the electromagnetic field varies in sinusoidal oscillatory way is in the time that in space. Under these conditions, electromagnetic radiation is represented by an **electromagnetic wave**.

All technologies, which use electricity for their operation, generate electrical fields, magnetic fields, or electromagnetic fields. However, it must be specified that for some technologies the generation of electromagnetic fields during their operation is neither intended or not necessary for the operation itself. For example, the purpose of a power line is not to generate electric and magnetic fields, but that of transporting and distributing electricity, so you can say that the electric and magnetic field are secondary effects not necessary for operation of the power line. This is typical of telecommunications systems where be transmitted remotely, must necessarily be supported by an emission of electromagnetic field. Non-ionizing radiation, also called N I R (Non Ionizing Radiation), has a frequency between 0 Hertz and 300 GHz (GIGAHertz) and can at them time to be divided into: magnetic fields at extremely low frequencies (ELF: extremely low frequency) ; radio frequency (RF); microwave (MO). In the range from 0 to 3000 Hz we have extremely frequency radiation low (Extremely Low Frequency, ELF) having a wavelength greater than 100 Km. This kind of waves are generated by low, medium and high voltage power lines, from distribution power lines and all devices powered by electricity to frequency of 50 Hz, for example household appliances, video terminals. At these frequencies very low the generated field is actually very similar to the union of two static fields, one electrical and other magnetic, rather than to a real electromagnetic field. [5]

2.2 Effect of the electromagnetic fields on biological systems

The biological effect of electromagnetic fields has been the subject of study since the beginning of nineteenth century and then leaved because of the complexity of matter. In the '50s interest resurfaced in countries such as the former Soviet Union, the USA, Italy and many studies were carried out mainly on the possible harmful effect of fields generated by power lines. Until now there is no general agreement on the exact effect of the fields electromagnetic on biological systems and in particular of extremely frequency fields low (ELF-EMF). The effects of exposure seem to depend on several factors such as the physical characteristics of the signal, the exposure parameters and the cell type, of consequence, the results from laboratories around the world are not always consistent with each other. The numerous data that have been obtained from in vitro e *in vivo* models, subjected to different experimental conditions, show that the magnetic fields of moderate intensity are able to influence different biological processes that are connected to the properties of the channels membrane.[6,7,8,9,10] These effects, initially studied by Liboff

[11,12,13], Blackman [14] and Lednev [15], were explained as an alteration in the calcium flow through the plasma membrane. Cellular mechanisms that are affected by exposure to ELF-EMF fields are numerous and include changes in the concentration of second messengers such as inositol 3 phosphate (IP3, Inositol Triphosphate) [16] , in calcium homeostasis [17,18] variations gene expression, proliferation [19,20,21], of cellular differentiation [22,23,24] and apoptosis. [25] The cellular models used to study the interaction between the biological system and magnetic fields were different. Among these we can mention the cells of the immune system such as the human lymphoid line [26,27], and the Human T cell line [28] ,epithelial cells such as human keratinocytes of the epidermis and oral mucosa [29,24],skeletal muscle cells such as the murine myoblasts that showed a change in the intracellular calcium concentration and cell proliferation, variations morphological accompanied by rearrangement of the cytoskeleton and expression of specific markers of differentiation. Interesting results were also achieved by studying the possible effects exposure to ELF-EMF on neuronal cells. Data show that exposure is able to stimulate the pathway that leads to cellular differentiation. This has been shown in rat chromaffine cells that differentiate in sympathetic neurons-like in following exposure to the field of 60 Hz, 0.7mt [30] and in PC12. [31,32,33] Further data from studies on neuronal progenitors where an increase was observed neurogenesis with an upregulation of the activity of the Cav1 channels. [34] Over the years, the researches have also seen the effect of the magnetic field on mesenchymal stem cells [35,36] by evaluating the proliferative and differentiation changes. Since the 1970s, experimental data have been obtained which highlighted in response to the application of electromagnetic fields, of appropriate intensity and frequency, an effect maximum biological at specific stimulation frequencies; this suggested the intervention of a new phenomenon called resonance. [37] An appropriate model for predicting how living organisms respond to magnetic fields would be to compare them to a radio receiver. In fact, a radio is immersed in a very rich environment of signals and waves, but it does not receive them all, because they are not at the frequency or at the appropriate modulation. But if we expose the radio to a signal properly tuned, this will interfere with the device, even if it is of very low intensity, and from it will be amplified. Similarly, if we expose a living system to a signal very weak electromagnetic, but properly tuned, this could interact in resonance with a normal biological function that develops weak currents endogenous alternates at that same frequency. [38] This kind of resonant interaction has been observed in many non-living systems, suggesting that weak magnetic fields can cause a resonance effect at the molecular level supra-molecular in electrolyte solutions, and therefore can thus affect in the biological processes. [39] A model based on the resonance phenomenon was proposed by Liboff in 1985 [11,12], to explain this aspect of the interaction between low frequency magnetic fields and biological systems, suggested that the cellular site of interaction could be membrane ion channels, precisely by virtue of their electrical characteristics. The ion transport through these channels is governed not only by voltage sensors, but also from oscillatory electric fields, which result from the helical distribution of carbonyl oxygens found in the canal walls on the luminal side. Liboff also proposed that the periodic changes in ion concentration are associated the oscillation of the Ca^{2+} ion could result in variations in the intracellular electric field. The resonance phenomenon would occur when the cell is exposed to a specific combination of low frequency alternating and weak static magnetic fields. According to Liboff, at the resonant frequency the ELF field and the static magnetic field would accelerate the molecules and ions in a spiral motion, for example inside a membrane channel, and this could alter the normal electrochemical physiology of the cell. Such

resonance phenomena could be not only the way in which external magnetic fields can interact with biological systems, but the same modality with which many cellular mechanisms work. [40]

2.3 Magnetic properties of materials

All the substances present in nature are characterized by different magnetic properties and a different interaction with external magnetic fields. Magnetic materials are those capable of becoming magnetized when subjected to the action of an external magnetic field. In these materials the movement of electrons around the positive nuclei of the atoms generates microscopic turns, the magnetic dipoles, crossed by electric current, with north and south poles. So, each atom that makes up the matter of magnetic materials is associated with its own magnetic dipole. When the dipole is immersed in an external magnetic field it orientates itself like the lines of force of the field. Consequently, depending on how magnetic dipoles behave in the presence of an external magnetic field, we have various categories of materials: **diamagnetic, paramagnetic, ferromagnetic, antiferromagnetic and ferrimagnetic.** The magnetization intensity vector, also called the magnetic polarization vector **M**, is the magnetic moment per unit of volume possessed by the material; for each substance it is possible to obtain a magnetic susceptibility, represented by the symbol **χm**, which indicates the ability of the substance to be affected by the actions of an external magnetic field; mathematically, susceptibility can be defined as the relationship between induced magnetization and the applied external magnetic field **H**: **$M = \chi m H$.** Based on this relationship, all substances occurring in nature can be classified. Different types of materials placed inside a solenoid show different magnetization effects. Diamagnetic materials ($\chi m<0$): experience shows that some materials are weakly repelled by the magnetic field produced by the solenoid. In this case, such as in water or silver, the atoms do not have their own magnetic dipole but generate it only when exposed to the magnetic field and it is oriented in the opposite way to the field. Paramagnetic materials ($\chi m>0$): the materials, such as aluminum and platinum, are attracted weakly by the magnetic field. Atoms have magnetic dipoles and in the absence of a magnetic field are randomly oriented along all directions of space; in the presence of an external magnetic field, on the other hand, they orient themselves along the lines of force of the field itself. Ferromagnetic materials ($\chi m>>0$): very strongly attracted to the solenoid, such as iron, nickel, magnetite, have their own magnetic dipoles organized in groups called **Weiss domains** in which the magnetic direction is uniform. (Fig.1)

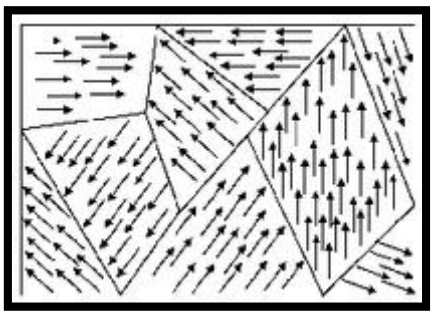

Figure 1. Weiss domains in a ferromagnetic material.

At a microscopic level, ferromagnetic materials have a more ordered structure so that even a not very intense external magnetic field is enough to ensure that all the magnetic dipoles are aligned uniformly, creating a much more

amplified overall magnetic field. In this case, the magnetic permeability constant χm assumes very high values depending on the temperature which, if it exceeds a limit value defined as the **Curie temperature**, causes the material to lose its ferromagnetic properties and behave like a paramagnetic material. Ferromagnetic substances also have the property of maintaining, after exposure to a magnetic field, their own residual magnetism as the Weiss domains tend to remain all oriented in the same direction. Each ferromagnetic material has its own magnetic hysteresis loop. (Fig 2) A hysteresis loop shows the relationship between the external magnetizing force and the induced magnetic flux density. The plot of Hysteresis is known as a B-H curve, where B (The Material's Flux Density, measured in Tesla or Mega Gauss) is plotted on the vertical axis and H (The External Applied Magnetizing Force, measured in Amperes per meter) is plotted on the horizontal axis. The Flux Density (B) of a magnetic material will increase in the presence of an applied magnetic field (H) until it reaches a maximum where it no longer responds to increases in the magnetic field. This is the point of Positive Saturation (Fig 2a), where any further increase in the external magnetic field will not increase the flux density of the material any further. The material releases some of its magnetism, and where the applied field has reached zero again, (point c on the B-H Curve) the material finds its residual magnetic strength, which is called Remanence (also known as Retentivity) (Fig 2b). Remanence is the remaining magnetic field found in the material after the applied magnetic field is reduced to zero. Based on the raw value of Remanence and the shape of the curve we are generating with the applied magnetic field; we can determine whether the specimen under study is a hard-or soft magnetic material. Another parameter that provides the hysteresis graph is the material's Coercivity (Fig 2c). Coercivity -also known as Coercive Strength- is the resistance of a material to changes in magnetization. Soft magnetic materials tend to have low coercivity and hard magnetic materials have high coercivity.

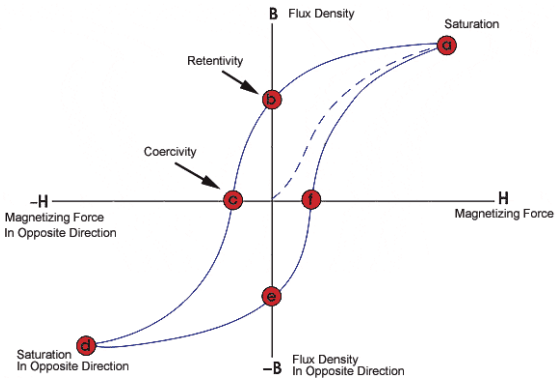

Figure 2. Hysteresis loop: a Positive Saturation; b Retentivity; c Coercivity; d Negative Saturation

When the spins of Weiss domains are oriented anti-parallel and of equal value, they compensate exactly we speak of anti-ferromagnetic materials. Antiferromagnetic materials occur commonly among transition metal compounds, especially oxides. Examples include hematite, metals such as chromium, alloys such as iron manganese (FeMn), and oxides such as nickel oxide (NiO). Finally, ferrimagnetic materials are those with anti-parallel spins, but which do not exactly compensate each other, giving the material a permanent magnetic moment, even without the application of a magnetic field. Ferrimagnetic materials have high resistivity and have anisotropic properties. The anisotropy is actually induced by an external applied field. When this applied field aligns with the magnetic dipoles, it causes a

net magnetic dipole moment and causes the magnetic dipoles to precess at a frequency controlled by the applied field, called Larmor or precession frequency. The oldest known magnetic material, Magnetite, is a ferrimagnetic substance. Other known ferrimagnetic materials include yttrium iron garnet (YIG); cubic ferrites composed of iron oxides with other elements such as aluminum, cobalt, nickel, manganese, and zinc. (Fig 3)

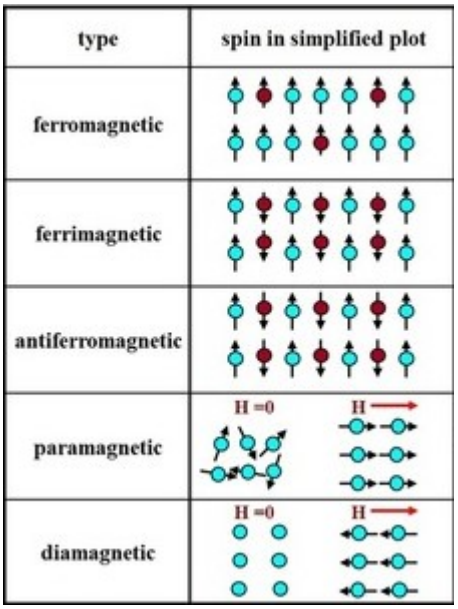

Figure 3. Magnetic moment arrangement scheme for different types of magnetic materials.

2.4 Magnetic nanoparticles (MNPs) and superparamagnetic nanoparticles (SPIONs)

Magnetic nanoparticles (MNPs) continue to sustain scientific interest due to their potential use in fields ranging from high-density data storage to biomedical applications [41,42,43]. The unique properties of MNPs derive from the fact that these nanoscale magnets differ from bulk materials due to their high surface-to-volume ratios. Different factors contribute to the control and optimization of the key magnetic properties of MNPs: saturation magnetization (Ms), coercivity (Hc), blocking temperature (TB), and relaxation time (tN and tB). The design of MNPs with tailored properties depends on the fundamental concepts of nanomagnetism (i.e., magnetism observed in nanoparticles). The studio on magnetic nanoparticles focuses on developing an optimal response for MNPs to an external magnetic field, typically classified as either ferrimagnetic, ferromagnetic, or superparamagnetic particles. Below certain critical dimensions, MNPs exhibit magnetic responses reminiscent of those of paramagnetic materials. This phenomenon, observed at temperatures above the so-called blocking temperature, arises from the thermal fluctuations within the nanoparticles being comparable to or greater than the energy barrier for moment reversal, allowing rapid random flipping of the nanoparticle magnetic moments. In the case where the magnetization of the MNP over the measurement/observation interval is equal to zero in the absence of an external field, such nanoparticles are referred to as superparamagnetic. For MNPs, the maximum magnetization possible is called the saturation magnetization, and it arises when all the magnetic dipoles are aligned in an external magnetic field. Although there is a strong dependence of magnetic properties on the size of the nanoparticles, magnetic behavior of MNP cannot be defined

with respect to one parameter, in fact there are other factors also can influence magnetic properties [44] how shape, composition, and shell-core design. A multitude of domains can be identified within a magnetic material, each of which is composed of a group of spins that point in the same direction and act at the same time. The formation of these domains is linked to the minimization of the system's energy. Their size is, in fact, dictated by the competition between the increase in energy imposed by the external magnetic field and the energy expenditure required for the formation of the "walls" that circumscribe the domains. These last they can be moved by applying an external magnetic field. Their movement involves an increase in the size of the domain oriented in the same direction as the applied magnetic field and gives rise to the magnetic hysteresis curve. The characteristic size of the domains depends on the material; it is usually in the order of tens of nanometers. When the size of the crystals becomes smaller than a single domain, they will behave from single magnetic domains, as forming additional subdomains becomes difficult. Such materials they have different chemical-physical characteristics than the bulk starting material [45]; in fact, the small size and the high surface area of the magnetic particles lead to a radical change in the magnetic properties: they, being single magnetic domains, will exhibit superparamagnetism phenomena [46]. In superparamagnetic systems the application of a magnetic field external causes a strong magnetization but following the removal of the external magnetic field no residual magnetic force remains between the particles [47]. Superparamagnetic nanoparticles, called SPIONs, show a high saturation magnetization, coercivity and zero residual magnetization [48]. Different types of magnetic materials, such as iron oxides (Fe_2O_3 and Fe_3O_4), metal alloys (Fe, Co and Ni), iron and cobalt alloys have been extensively studied for magnetic drug delivery systems. Among these materials, magnetite (Fe_3O_4, single domains of 5-10 nm), the common magnetic iron oxide, is the ideal candidate for its biocompatibility and biodegradability. it has in fact been shown that SPIONs, after *in vivo* administration, can be metabolized and that the iron ions released add up to the amount of iron present in the body and incorporate the hemoglobin of the erythrocytes and therefore degraded with the normal recycling path of the iron. [49,50]

2.5 Applications of SPIONs in the biomedical field

In 1970 Freeman and his research group introduced the concept of magnetism in the medical field [51]. Since then, research has yielded different types of magnetic particles and different ways of using them by optimizing their properties. SPIONs are synthetic particles made up of: maghemite (γ-Fe_2O_3), magnetite (Fe_3O_4) and hermatite (α-Fe_2O_3); they have a core with a diameter between 10 and 100 nm and have a coating on which or inside which the drug can be loaded. The peculiarity of these systems is in fact that they can be conveyed to the target tissue through the application of an external magnet. Being equipped with superparamagnetism, the SPIONs are magnetizable up to saturation, in the presence of a magnetic field applied from the outside; when the field is removed, the nanoparticles will not show no residual magnetization. This property is a function of the size of the nanoparticles and usually takes over for values of at least 10-20 nm in diameter. Below these values the system does not have the multiple domains that are instead present in all the larger magnets and on the contrary behaves like a "single super spin" with high magnetic susceptibility. In superparamagnetic systems, after removal of the field, the nanoparticles are redispersed, making the formation of aggregates less likely, which could limit the half-life time of the system as it would be more easily engulfed and eliminated if it were in the form of aggregates. The SPIONs, for their physical,

chemical, thermal and magnetic, have a large number of potential biomedical applications such as cell labeling / cell separation; tissue repair; magnetic resonance imaging (MRI); hyperthermia; magnetofection; treatment of arthritis and drug delivery. The labeling of cells with iron / paramagnetic substances is a method used for the separation of cells *in vivo* and to ensure that the labeled cells are identified by MRI. *In vivo* cell labeling and separation techniques use two approaches: attachment of magnetic nanoparticles on the cell surface [52] or internalization of biocompatible magnetic nanoparticles through fluid phase endocytosis [53], receptor mediated endocytosis [54] or phagocytosis [55]. A strategy for effective and specific cell marking with magnetic nanoparticles consists in modifying the surface of the nanoparticles with specific ligands, in such a way that the cell does not it only recognizes the system but internalizes it through receptor-mediated endocytosis [56]. The SPIONs, suitably functionalized with organic molecules, can act as markers of specific cells *in vivo*; they can be used, for example, to identify tumor areas especially in the liver and lymph nodes. Another application is to functionalize the SPIONs with specific ligands for some biomolecules in order to act, for example, as a DNA marker to identify "wrong" sequences. Tissue repair involves transplanting stem cells into a injured tissue, which growing, proliferating and differentiating repair tissue damage. In this field the SPIONs have been proposed as tools to support stem cells and allow their delivery to desired sites. If the stem cells are labeled with SPIONs, it makes it easier to trace them inside the body using, for example, MRI. In doing so, in fact, it will be possible to follow the path of stem cells *in vivo* without using invasive techniques [57,58]. The advantages that SPIONs offer are biocompatibility, as evidenced by the fact that the use of SPIONs as contrast agents has been approved by the Food and Drug Administration; the magnetic susceptibility of the nanoparticles makes the whole system a good contrast agent for MRI; moreover they can undergo superficial modifications in order to allow the anchoring of different types of stem cells. Superparamagnetic nanocrystals of iron oxides can be used as contrast agents in MRI (Magnetic Resonance Imaging), a technique used in the medical field that measures the relaxation of proton nuclear spin in tissues, managing to transform the signal into an image. Micrometric magnetic particles or chelated complexes of magnetic ions injected into tissues act as proton relaxation agents. Magnetic nanocrystals have the advantage of being able to circulate in the blood for a longer time than larger crystals, to be able to cross the capillary walls and reach the lymph nodes and also to be more effective proton relaxation promoters [47]. The use of hyperthermia (heat) in the treatment of malignant tumors is one very old technique. The magnetic field is not absorbed by living tissues and can be applied to deep regions of the body. When magnetic particles are subjected to an alternating magnetic field, heat is generated due to the magnetic loss of hysteresis. The amount of heat generated depends on the nature of the magnetic material and the parameters of the field. The magnetic particles incorporated inside the tumor cells subjected to an oscillating magnetic field, heat up, reaching a temperature that depends on the magnetic properties of the material, the strength of the magnetic field, the frequency of oscillation and the cooling capacity of the blood flow in the site where the tumor is present. Therefore, as cancer cells are sensitive to high temperatures will be destroyed when the temperature reaches values close to or above 43 ° C, while normal cells, more resistant to high temperatures, will survive [59]. Magnetofection is a technique in which nanoparticles superparamagnetics associated with DNA vectors are transfected inside the cells through the application of an external magnetic field. For this purpose, superparamagnetic nanoparticles must be coated with polycations, such as polyethylene (PEI) [60]. SPIONs, through the endosomal barrier, release the

gene of interest into the nucleus. Using permanent or alternating magnets the nanoparticles will be forced to follow the magnetic field avoiding free diffusion. Corticosteroids represent a solid standard in arthritis therapy. They are injected directly into the affected joints; although are able to treat the symptoms of this pathology, over time they can cause the formation of crystals that cause joint infections. Recently, systems based on SPIONs e corticosteroids, and it has been shown that they can be useful in solving the problem. Through the application of an external magnetic field, these systems can be retained in the joint capsules avoiding repeated injections and elimination due to macrophage and drainage systems [61]. Finally, an important application of SPIONs provides for the specific distribution of the drug, drug delivery systems (DDS), at the site of action. The functionalization of the surface of the SPIONs makes them excellent drug carriers that are thus directed into the organ target and released there. In fact, by applying an external magnetic field it will be possible to direct the drug delivered to a specific site, thus reducing the doses to be administered and the potential harmful side effects [62,63,64]. Properly designed carriers can be applied in numerous pharmaceutical fields, for oral administration, for sustained release, for drug targeting at specific sites, and for parenteral administration of anticancer drugs. For such application, the size, charge and surface chemistry of the magnetic particles are very important, as they affect the circulation time in the blood and the bioavailability of the particles within the body. [65]

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Chapter 3 Effect of magnetic field on periodontal ligament stem cells

3.1 Introduction

Periodontal ligament stem cells (PDLSC), being easily available, multipotent, with a high self-renewal capacity and consistent immunomodulatory effects, phenotypically and morphologically similar to mesenchymal stem cell (MSCs), [1,2,3] represent an ideal source of stem cells for the development of cell therapies and tissue engineering applications. [4] Seo et al. in 2004 isolated, for the first time, PDLSC from impacted human third molars and discovered their ability to differentiate into periodontal bones, cementum, alveolar ligaments, peripheral nerves and blood vessels. [5,6,7] It is well known that PDLSCs have an important role in maintaining periodontal homeostasis and are responsible for regeneration and remodelling of periodontal tissues. [7,8] Various studies have clarified that mechanical forces, such us vibration, compression, and tension, can significantly regulate the proliferation and differentiation of cultured PDLSCs, in vitro. [9] But in the medicine regenerative is well known that in addition to mechanical strain, other biophysical stimuli such as ultrasound and electromagnetic fields can to improve tissue regeneration. [10,11,12] There is a long history of clinical use of static and pulsed magnetic fields in orthopedic field to increase bone fractures healing, to improve osseointegration of implants or to treat congenital pseudoarthrosis. [13] Furthermore magnetic field has been used in studies concerning drug delivery, hyperthermia for cancer, magnetic cell constructs stimulation and control of cell proliferation and differentiation. [14,15] Several studies in the literature show how the stimulation with a specific magnetic field of a biocompatible material may bring important advantages in terms of cell-material interaction. [16,17] Biochemical properties of stem cells and different cell populations may be influenced using external static or pulsed magnetic field. Wang et al in 2017 [18] showed that PDLSCs respond to a pulsed electromagnetic field (PEMF) stimulation improving the osteogenic properties but no the cell proliferation. It is accepted also that magnetic stimulation leads to different effects based on some different parameters such as waveform, frequency, intensity, duration and type of cell. [19] However, the mechanisms underlying PDL cell property changes induced by magnetic field are still unclear. For these reasons, we have decided to investigate the effect of a specific discontinuous time-dependent magnetic field (6 h per day with 20 intervals of 18 min each) [20] on the PDLSCs biological behaviour, evaluating variations on cell proliferation, differentiation and metabolic respiration.

3.2 Materials and Methods

3.2.1 Cell culture

Periodontal Ligament Stem Cells (PDLSCs) were obtained from different patients aged 19–25 years (mean age ¼ 22.7 years). After the surgical extraction, impacted third molars were collected. PDL was collected by scraping the root surface from the middle third to the apical third, without involving apical papilla, and then

minced. Small pieces of tissue were digested using 2 mg/ml collagenase type I solution (Gibco, Life Technologies, Italy) for 2 hours at 37 °C. PDLSCs obtained from three PDLs of the same patient were seeded into a plate with culture media and incubated at 37 °C in a humidified atmosphere of 5% CO_2 to growth. The cells, characterized by flow cytometry, showed positivity for CD73, CD90 and CD105 while they are negative for CD14, CD45, CD34. [21] PDLSCs, at the fourth passage, were cultured in Dulbecco's modified eagle medium high glucose (DMEM, Sigma Aldrich, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 200 mM L- glutamine (Euroclone,Italy) and antibiotics (penicillin G sodium 100 U/mL, streptomycin 100 mg/mL, Euroclone,Italy). The cells, seeded on cell culture dishes, were incubated in a humidified atmosphere at 37 °C and 5% CO_2. The cells were subcultured using trypsin/ethylene diamine tetraacetic (EDTA, Sigma Aldrich, Germany).

3.2.2 Magnetic field stimulation

Periodontal ligament cells have been stimulated with a time-dependent magnetic field. One day after cell seeding an external sinusoidal magnetic field with a frequency of 70 Hz and intensity of 30 mT was discontinuously applied for 6 h per day with 20 intervals of 18 min each. [20] Incubator equipped with an electromagnet was used; cell plates were placed over the electromagnet to expose cells to the magnetic field. To avoid mutual influence, the plates have been placed at the right distance between them, about 10 mm. The non-magnetically stimulated plates used as control were placed inside the same incubator.

3.2.3 Western blot analysis

PDLSCs, with a density of 1.2 x 10^5cells, were seeded in a 60 mm culture dishes. Two and four days after magnetic stimulation (6 h per day for 20 intervals of 18 min each), cells were serum-deprived overnight and left untreated or treated with fibroblast growth factor (FGF2—10 ng/mL; Sigma) with treatment times of 30 min. The cells were collected with trypsin, centrifuged, and were obtained pellets. After pellets were lysed in a saline buffer containing 1 mM EDTA, 50 mM Tris-HCl (pH 7.5), 70 mM NaCl, 100 mM NaF and 1% Triton. The lysates were cleared by centrifugation at 15,000 g for 10 min and quantified by the Bradford method. Lysed cells aliquot were resolved on sodium dodecyl sulfate polyacrylamide gel and transferred on nitrocellulose membrane for 3 h. Filters were blocked for 1 h at room temperature in Tween-20 Phosphate buffer saline (TPBS) (PBS- Sigma, 0,1% Tween 20, pH 7.4) containing 5% non-fat dry milk. Blots were then incubated O/N with primary antibody. They were washed three times with a TTBS buffer and incubated for 1 h with a secondary antibody (peroxidase-coupled anti-rabbit) in TTBS-5% (w/v) non-fat dry milk. Reactive signals were detected by an ECL Western blotting analysis system. The autoradiography images were acquired and analyzed with Image-J software (NIH, Bethesda, MD, USA). The following primary antibodies were used: rabbit Phospho ERK (Thr202/Tyr204) (Cell Signalling), rabbit ERK1/2 (Santa Cruz). Polyclonal antibodies were used at working dilutions of 1:1000.

3.2.4 Cell proliferation assay

To evaluated proliferation and viability cell of PDLSCs stimulated and unstimulated with magnetic field was used the Alamar Blue assay (AbD Serotec Ltd., UK). Periodontal Ligament Stem Cells (PDLSCs), at the fourth passage, were seeding in a 48 multiwell plates using a density of 1.0 x 10^4 cells. At 4, 7, 14, and 21 days after cell seeding, the cells were rinsed with PBS (Sigma–Aldrich, Italy) and 200 µL of DMEM without phenol red (HyClone, UK) containing 10% (v/v) Alamar Blue was added for each sample. The samples were incubated for 4 hours in 5% CO_2 diluted atmosphere at 37 ◦C. Subsequently, 100 microliters of the solution were removed from the wells and transferred to a 96-well plate. The assay is based on a redox reaction in the mitochondria of the cells; the coloured product is transported out of the cell and the optical density is spectrophotometrically measured at wavelengths of 570 and 595 nm. (Sunrise; Tecan, Mannedorf, Zurich, Switzerland). This experiment, like all the others, was performed in triplicate.

3.2.5 Osteogenic differentiation assay

Alkaline Phosphatase (ALP) is an important marker for the early state of osteogenic differentiation in stem cells. A specific enzymatic assay (SensoLyte pNPP alkaline phosphatase assay kit - AnaSpec Inc., Fremont, CA, USA) was used to evaluate the ALP activity. This assay is based on based on the substrate p-nitrophenyl phosphate (pNPP). PDLSCs were seeded into 48-well plates (density of 1.0 x 10^4 cells) and subjected to magnetic exposure. A non-magnetically treated multiwell plate was used as a control. At 7, 14 and 21 days after the seeding, the cells were washed twice in PBS and lysed in 1 ml of lysis buffer. After collecting and centrifuging, the supernatant was used to calculate alkaline phosphatase (ALP). After 30-minute incubation with pNPP, the phosphatase is completely inhibited by NaOH and the pNPP liberated form a yellow anion. The ALP activity is directly proportional to the amount of pNPP liberated per unit time.

3.2.6 Metabolic activity assay

Periodontal ligament stem cells PDLSCs, at fourth passage, with a density of 1.2 x 10^5 cells, were seeded in cell culture flasks. After 4 days of magnetic stimulation (6 h per day for 20 intervals of 18 min each) the cells, at the density of 6.0 x 10^4 cells per well, were reseeded in triplicate into specific cell culture microplates (Agilent, USA) to be analysed with two different metabolic kits: Seahorse XF Real-Time ATP Rate Assay Kit and Seahorse XF Cell Mito Stress Test Kit (Agilent, USA). Under the same conditions, cells not treated with the magnetic field were also analysed. After 24 h, basal OCR was measured four times and plotted as a function of cells under the basal condition followed by the sequential addition of oligomycin (1 µg/ml), FCCP (1 µM), and rotenone (1 µM).

3.2.7 Confocal Laser Scanning Microscopy images

For immunofluorescence study, PDLSCs cells, treated and untreated with magnetic field for 4 days, were plated on poly-l-lysine coated (10 μg/ml) glass coverslips, fixed, and immunostained with the primary antibody HAHDA. The immunoreactive signals were visualized by fluorescent-labeled secondary antibodies. The fluorescent signals were visualized using a Zeiss LSM 510 Meta argon/krypton laser scanning confocal microscope. Confocal images were acquired using a LSM 700 Zeiss confocal microscope (Carl Zeiss International, Germany). Images were captured using the ZEN software (Carl Zeiss International).

3.2.8 Statistical Analysis

All experiments were independently repeated 3 times. The data were represented as means ± standard deviations. 1-way analysis of variance (ANOVA, Bonferroni post hoc test) was applied for multiple groups. A value of $p < 0.05$ was defined as statistically significant.

3.3 **Results**

3.3.1 Effect of magnetic field on cell proliferation

The percentage of reduction of Alamar Blue, at indicated times, show a good cell viability both in MAG treated and untreated cells, with a maximum peak at 7 days. In addition, 21 days after cell seeding, a significant increase ($p < 0.05$) in the cell proliferation was found for PDLSCs MAG compared to the control cells. (Fig 1a) Western blot analysis show FGF-dependent ERK activation. (Fig 1 b,c) Remarkably the presence of magnetic field is sufficient to stimulate a robust phosphorylation of ERK1/2, both in presence and in absence of FGF stimulation, compared to control (Fig 1b, c). The ERK1/2 phosphorylation levels are dependent on the time of MAG treatment, as shown by the increase of phospho-ERK1/2 levels between 2 and 4 days of exposure, nonetheless the FGF stimulation of MAG-PDLSCs still cause a further activation of ERK1/2.

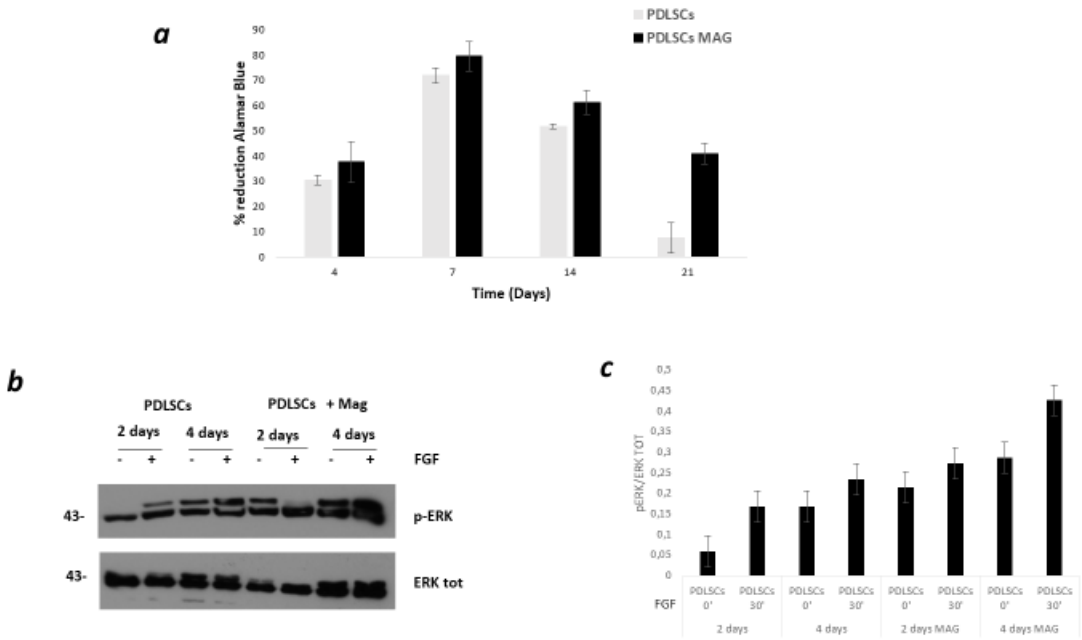

Figure 1. **(A)** Percentage of reduction of Alamar blue evaluated for PDLSCs and PDLSCs MAG at different time points. **(B)** Western blotting analyses performed on PDLSCs stimulated with a time-dependent magnetic field or left unstimulated. The experimental groups were treated with fibroblast growth factor (FGF2) at indicated time points (0 and 30 min). **(C)** Quantization of p-ERK expression normalized with ERK tot in cells treated with and without magnetic field after 2 and 4 days.

3.3.2 Effect of magnetic field on cell differentiation

The osteogenic differentiation was evaluated through the measurement of the ALP levels (ng/ml) over time. The histogram shows a higher increase of ALP at 14 days after the cell seeding for the magnetically-stimulated cells (PDLSCs MAG) if compared to the unstimulated ones. (Fig.2) The decrease in ALP activity from the day 14 to day 21 is attributed to reaching a plateau of ALP expression and the onset of an advanced stage of osteogenesis indicated by later osteogenic markers.

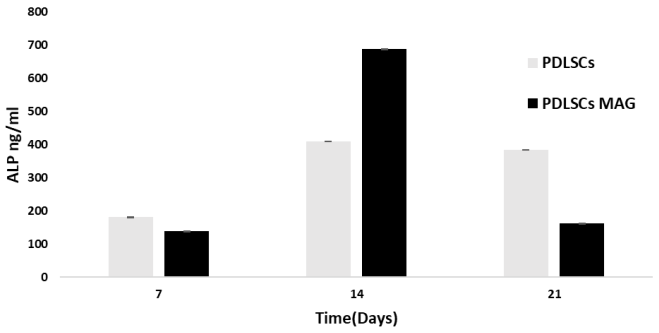

Figure 2. Alkaline phosphatase (ALP) activity for PDLSCs and PDLSCs MAG at 7, 14 and 21 days after cell seeding.

3.3.3 *Effect of magnetic field on cell metabolism*

Oxygen consumption of cells was determined using the Seahorse analyser. Data are showed as oxygen consumption rate (OCR) in pmoles/min protein and extracellular acidification rate (ECAR) in mpH/min protein. The results show a significant reduction in baseline oxygen consumption of

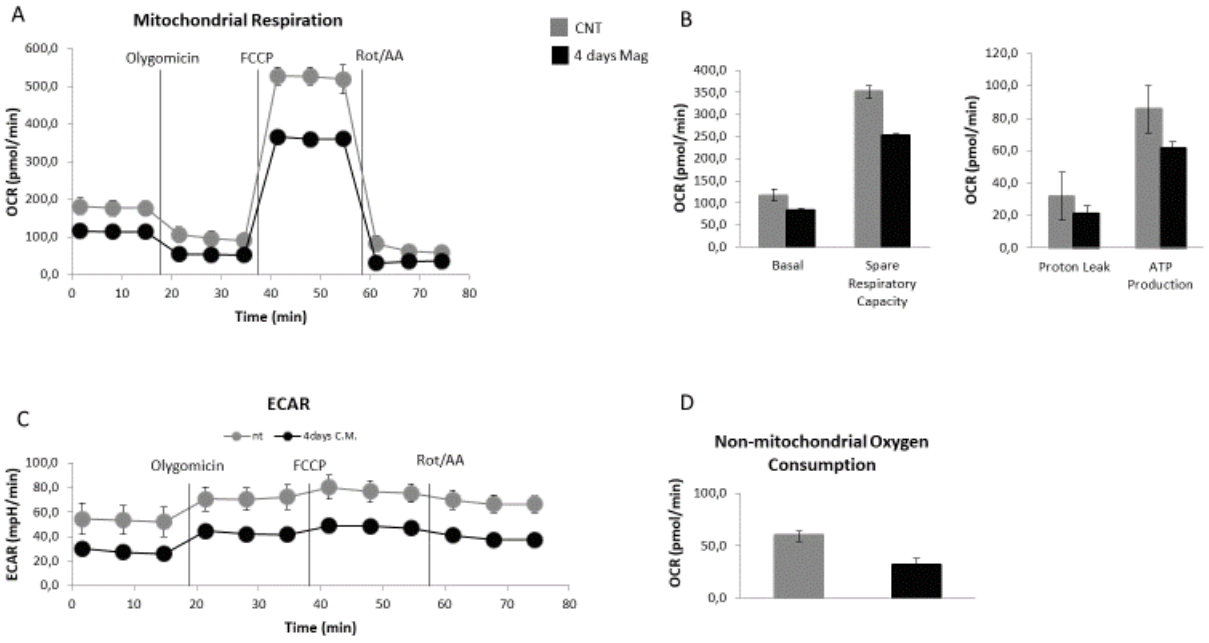

Figure 3. Respiration assays. **(A)** Mitochondrial respiration measured with sequential additions of oligomycin (1.5 μM), FCCP (1 μM), rotenone (3.0 μM). **(B)** Graphs represent the OCR rate. **(C)** ECAR rate. **(D)** Non-mitochondrial Oxygen consumption rate.

cells exposed to the magnetic field compared to the control cells. DPLSCs stimulated with magnetic field show a decreased ECAR level, a lower non- mitochondrial oxygen consumption and consequently a minor ATP

production. (Fig 3) Considered the decreased Oxygen consumption rate of MAG treated cells, we decided to monitor the glycolitic asset of the treated cells compared to control. Figure 4 shows ATP production rates from mitochondrial respiration and glycolysis in real time. (Agilent Seahorse) This assay is able to detect mitochondrial Oxygen Consumption Rates (OCR) and Proton Efflux Rate (PER) which are then transformed to mitoATP and glycoATP production rate. This assay allows us to quantify metabolic changes in response to a discontinuous magnetic field. The data shows a slight decrease of glycolitic ATP production of PDLSCs MAG over control cells, and a dramatic decrease of mitocondrial ATP levels of cells treated with magnetic field.

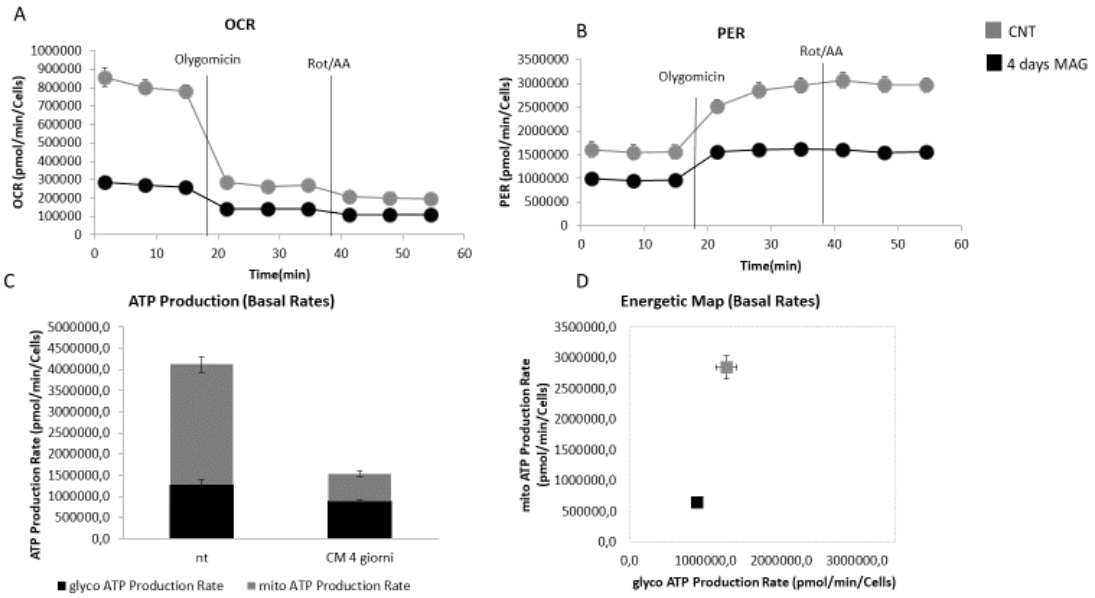

Figure 4. ATP rate assays. **(A)** Analysis of O2 consumption. The rates of oxygen consumption (OCR) were measured with sequential additions of oligomycin (1.5 μM), rotenone (3.0 μM). **(B)** Proton Efflux Rate (PER). **(C)** ATP production rate. **(D)** Energetic maps.

3.3.4 Mitotracker uptake is impaired due to MAG exposure

The severe reduction of OCR followed by the decrease of mitochondrial ATP production after MAG treatement, let us wonder if mitochondria physiological state was affected by MAG exposure. To this aim we exposed PDCSCs to 4 days of Magnetic field and checked mitochondria state trough two different markers. Mitotracker is a red-fluorescent marker that stains mitochondria of living cells, dependent on mitochondrial membrane potential. MAG affects the membrane potential of mitochondria as shown by the lack of retention of the dye in MAG treated cells, that results in a lower expression of red fluorescence. To verify that mitochondria of PDCSCs exposed to MAG were still healthy we performed the same experiment with a different mitochondrial marker, HADHA, a member of fatty acid beta-oxidation, that localizes in mitochondrial matrix.The staining of HADHA in MAG treated cells, shows that, despite the alteration of

mitochondrial membrane potential, the mitochondrial morphology seems to be unaffected by MAG treatement (Fig5).

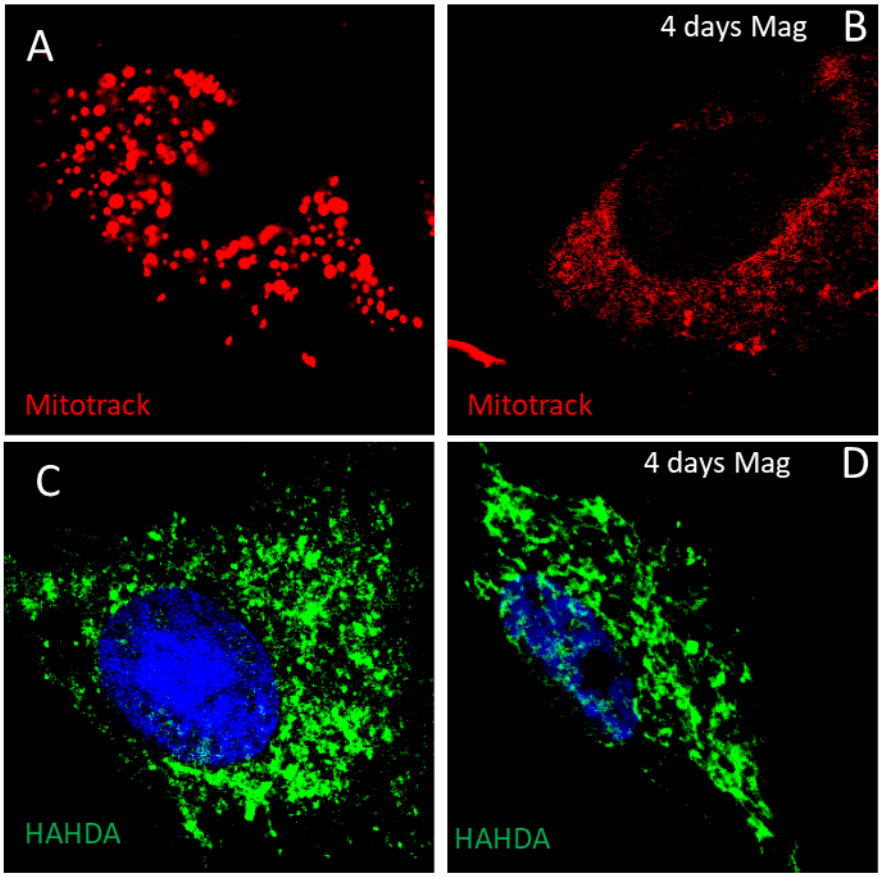

Figure 5. Confocal laser scanning microscopy analysis on PDLSCs 4 days magnetic stimulation later. **(A-B)** Images with mitotracker fluorescence (red) of PDLSCs magnetically unstimulated and stimulated. **(C-D)** PDLSCs immunostained with the antibody HAHDA (green) without and with magnetic exposure. Scale bar: 100 µm.

3.4 Discussion

In this study, we provided a novel approach to the periodontal regeneration. Synergic effect of a biophysical external stimuli such as a time-dependent magnetic field in combination to the osteogenic regeneration potential of PDLSCs performed a primary data to future application also in the biomaterials area. Starting from the known MSC-like biological features of periodontal ligament stem cells,[22] we assessed the changes that a magnetically stimulation carry out in terms of proliferation, differentiation and cell metabolism. In literature is showed that PDLSCs respond to PEMF stimulation, [18] electromagnetics field different from used in this research, but there are several contradictory still unclear effects on cellular behaviour [23,24] depending on different magnetic field parameters (intensity, frequency, exposure time) used. In this scenario, we investigated the main pathway involved on cells proliferation, apoptosis and differentiation: the mitogen-activated protein kinase family (MAPK) cascade. [25,26] We checked the cellular vitality of PDLSCs over MAG treatment

through Alamar blue assay. Therefore, to investigate the effect of time-dependent magnetic field on periodontal ligament stem cells (PDLSCs MAG), we monitored the ERK1/2 phosphorylation on Thr202/Tyr204 in course of stimulation with FGF, a well- known activator of MAPK cascade. (Fig6)

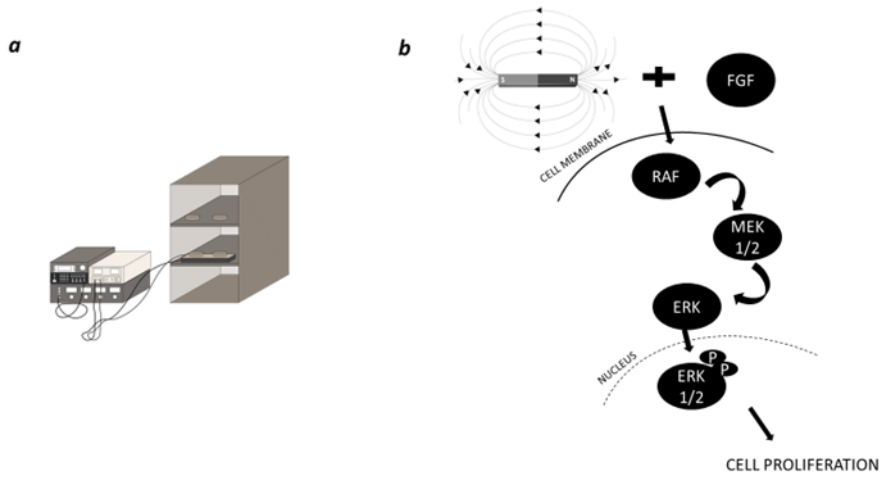

Figure 6: (A) Illustration of the magnetic device and of the incubator equipped with electromagnet. (B) Schematic diagram illustrating the MAPK signaling pathway triggered by exposure to magnetic field.

As previously mentioned, MAPK cascade is involved in numerous cell activity, included cell differentiation. To investigate whether the increased activation of ERK, caused by MAG exposure, is also affecting PDLSCs osteogenic expression, we monitored the expression level of ALP. Alkaline Phosphatase (ALP) is one of the most reliable markers for early osteogenic differentiation, as it is produced by osteogenic cells such as osteoblasts or by stem cells capable of osteogenic differentiation. [27] Moreover, considering the remarkable effects on cell growth and differentiation caused by MAG exposure on PDLSCs, we decided to investigate the metabolic state of cells exposed to MAG for 4 days.

The results showed a good cell vitality and a major proliferation activity of PDLSCs cells stimulated with magnetic field compared to the control cells. With or without FGF, ERK activation is observed in presence of magnetic field but the presence of FGF together with a magnetic exposure cause a greater activation of ERK1/2. So, we postulated that time-dependent magnetic field (70 Hz, 30 mT) interacts with cell proliferation activating the MAPK pathway. The significant increase in the ALP activity in PDLSCs exposed to MAG for the indicated times compared to cells without the magnetic stimulation, suggests that the magnetic field induces the periodontal ligament cells to differentiate more. This data allowed us to assume that an external magnetic field induces the periodontal ligament cells to differentiate more. Benefiting from the previous data, we wondered how it was the metabolic state of PDLSCs stimulated by a discontinuous magnetic application. Our results show that a magnetically stimulation causes a decrease of oxygen consumption, a decreased of extracellular acidification level and a minor ATP production, mainly mitochondrial ATP. But nevertheless it,

the PDLSCs MAG mitochondria, observed under confocal microscope, do not appear morphologically damaged. Getting to know these metabolic data is important as energetic changes in cellular metabolism during glycolysis or OXPHOS oxidative reactions (states of hypoxia, glycolysis and redox) have an impact on stem cell differentiation, reprogramming, [28,29] homeostasis and regeneration. [30,31,32] Hence understanding the metabolic mechanism of PDLSCS stem cells, in our case in combination to a time-dependent magnetic stimulation, is critical to facilitate their use in regenerative medicine by increasing the control of their manipulation in vitro and promoting their migration into tissue damage. [33] MAPK cascade represent a fundamental mitogenic pathway that also controls metabolism, differentiation and cell growth.

3.5Conclusion

In conclusion, use of a sinusoidal time-dependent magnetic field, used as an external biophysical stimulus, determines relevant variations of periodontal ligament stem cells PDLSCs biological behaviour, improving their proliferative and differentiation capacity. In doing so, is promoted their dental regenerative application, and not only. Future studies will investigate the cell-material interaction of magnetically exposed PDLSCs.

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Chapter 4 Injectable hydrogel for tissue engineering and regenerative medicine

4.1 Introduction

Typically, tissue engineering strategies involve three critical elements: stem cells or progenitor cells, signalling molecules (e.g., growth factors), and scaffolds. The scaffold is an artificial extracellular matrix (ECM) and serves as a template for cell growth and tissue regeneration. Ideally, the scaffold should be biocompatible and biodegradable, should possess proper mechanical and physical properties, and mimic the *in vivo* microenvironment (niche) to facilitate cell adhesion, proliferation, differentiation, and tissue formation [1].

Based on when a scaffold is shaped, it can be either a pre-formed or an injectable scaffold. A pre-formed scaffold has a definite shape prior to its application, while an injectable scaffold is shaped *in situ.* Compared to pre-formed scaffolds, injectable scaffolds present several advantages, including (1) being performed in a minimally invasive manner, therefore decreasing the risk of infection and improving comfort; (2) they can easily fill irregularly-shaped defects; and (3) they overcome the difficulties of cell seeding and adhesion, and the delivery of bioactive molecules, as these factors can simply be mixed with the material solution before being injected *in situ*. [2] This study starts from the need to evaluate the regenerative capacity of a biomaterial loaded with stem cells extracted from the periodontal ligament, if introduced into a mandibular bone damage of mice. Therefore, due to the size, morphology and complicated structure of the damage to the dental or craniofacial tissue, an injectable scaffold is more advantageous than a preformed one. A variety of biomaterials have been proposed for use as injectable scaffolds. According to the source of origin, they can be classified as natural and synthetic biomaterials. Natural biomaterials are derived from natural resources and have the advantage of biological recognition, which may positively support cell adhesion and growth. These materials usually are biocompatible and biodegradable, and do not cause inflammatory or immune responses. [3] However, there are concerns with natural materials regarding potential pathogen transmission and the variability of quality from batch to batch. These concerns have led to a vast amount of research in the development of synthetic biomaterials as substitutes for naturally derived ones for tissue engineering purposes. Synthetic biomaterials can be manufactured on a large scale with well-controlled properties of strength, degradation rate and microstructure. Furthermore, synthetic biomaterials avoid the risk of pathogen transmission associated with natural ones. [4] However, synthetic biomaterials usually do not provide biological cues in their molecular chains, and the conjugation of specific cell-recognizable signal molecules (motifs) is often needed to facilitate cell-material interaction. Another way to improve the overall performance of a biomaterial is the use of a composite biomaterial, which combines the advantages of its individual components.

In addition, in recent years, the interest in the effects of static magnetic fields (SMFs) and non-ionizing electromagnetic fields (EMFs) on biological organisms has increased considerably. However, contradictory effects are described in different studies [5,6,7]. SMFs are widely used in clinical settings for a variety of

conditions, including bone fracture repair, migraines, skin ulcers and degenerative nerve disorders [8]. The US Food and Drug Administration approved the use of EMFs in clinical practice for treating non-union bone fractures and for osteoporosis therapy [9]. Animal studies also shown that SMFs increased bone strength and reduced inflammation [10,11], and they have been successfully used for treatment of bone fracture malunion [12]. Lim et al. (2009) [13 20] summarized the major effects of moderate-intensity SMFs on cultured cells and concluded that the diverse results were due to different exposure times and cell types. Static magnetic fields (SMFs) enhance the proliferation of several cell types.

The recent use of magnetic nanoparticles (MNPs) in various diagnostic and therapeutic applications has triggered the development of novel three-dimensional (3D) magnetic gradient and injectable gel scaffolds that can play an important role in cell signalling by guiding cell migration, proliferation, and differentiation. [14,15] MNPs have been widely used in biomedical applications due to their biocompatibility, large surface functionalization feasibility, tuneable physicochemical properties, and ability to interact with an externally applied magnetic field. [16,17].

The aim of this part of research was carried out preliminary study in vitro for a forward *in vivo* application.

Hydrogel matrix (Collagen /Hyaluronic acid) with a previously determinate magnetic nanoparticles (Fe_3O_4) concentration and PDLSCs (periodontal ligament stem cells) as cellular lines was performed to study the effect of magnetic field on the selected matrices with or without magnetic nanoparticles. In vitro tests of adhesion, proliferation and osteogenic differentiation on selected matrices were conducted. The optimal magnetic nanoparticles concentration was evaluated through a biological cell viability test (Alamar blue assay). The AlamarBlue assay showed information on cell viability and proliferation over time through a quantitative evaluation of the reduction percentage of AlamarBlue for cell-laden hydrogel. Preliminary confocal laser scanning microscopy was carried out to study cell adhesion and spreading on the injectable nanocomposite hydrogel over time. The ability of nanocomposite hydrogel to promote the osteogenic differentiation of the cells was assessed by the Alkaline phosphatase test (ALP test). Each of these tests were processed in with or without a time dependent magnetic stimulation.

4.2 Material and methods

4.2.1 Synthesis of Collagen/Hyaluronic acid Hydrogel

Rat tail collagen high concentration (10 mg/ml) (BD Biosciences, USA) and Hyaluronic acid (Sodium Hyaluronate grade HMW (10 gr) Altergon, Italia) was used to obtain the nanocomposite Hydrogel.

Collagen-HMW HA NPs hydrogel were obtained by suitably adding HMW HA, dissolved in DMEM no phenol red (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), to the sterile collagen solution to provide a final HMW HA concentration of 2.5 mg/mL and collagen concentration of 1.2 mg/mL. Polyvinylpyrrolidone (PVP)-coated Fe_3O_4 (99.5%, 25 nm, 0.2 wt % PVP, NanoAmor, Houston, TX) nanoparticles were added to the mixture to the final concentration of 25 µg/ml. Nanoparticles were used at different concentrations (50, 25,

10 µg/ml) before obtaining the ideal one. All steps were performed under sterile conditions. In the mixture was added PDLSCs periodontal ligament stem cells, at four passage, with a cellular density of 60.000 cells/ml. The solution was incubated at 37 °C for 1 h to promote collagen fibrillogenesis. After the incubation the hydrogel-based composite system was obtained.

4.2.2 Magnetic stimulation

A time-dependent magnetic stimulation of Hydrogel was performed at 1 day after cell seeding. An external sinusoidal magnetic field was discontinuously applied for 6 h per day (20 intervals at 18 min each) over time [46]. Further cell-laden Hydrogel with and without NPs were used as controls because they were placed in the same conditions without magnetic stimulation.

4.2.3 Biocompatibility and cell proliferation assay

Alamar blue assay was used to evaluate firstly the ideal concentration of nanoparticles in the hydrogel without HA (COL+NP) and with HA (COL/HA+NP). Successively, the alamar blue assay was used to estimate the effect of magnetic field on periodontal ligament stem cells proliferation. Cell proliferation was evaluated after 2,5,7,14, and 21 days to the cell seeding by using the cell viability reagent Alamar Blue (AbD Serotec Ltd., UK). The cell seeded hydrogel were rinsed with PBS (Sigma–Aldrich, Italy). The reagent was added to the culture medium DMEM without phenol red (HyClone, UK) at a concentration of 10% v/v to each well. Cells-sample constructs were then incubated for 4 hours at 37 ◦C in the incubator. The supernatant was removed in 96-well plate and its absorbance was quantified by spectrophotometry at 570 and 595 nm. The levels of cell proliferation were expressed as percentage with respect of the control. The experiments were done in triplicate.

4.2.4 Differentiation assay

Alkaline Phosphatase Level Test was performed to evaluate the ability of the periodontal ligament stem cells to differentiate in an osteogenic sense when they are seeded in hydrogel and magnetically stimulated. Prior to analysis, samples were manually homogenized, passed through three freeze-thaw cycles. Activity of ALP, an osteogenic marker produced by differentiating stem cells, was assessed with colorimetric assay using p-nitrophenyl phosphate (pNPP) substrate in alkaline buffer solution. (SensoLyte pNPP alkaline phosphatase assay kit - AnaSpec Inc., Fremont, CA, USA).

4.2.5 Confocal Microscopic assay

Cellular adhesion, morphology and cell spatial distribution into the 3D hydrogel samples were verified 2 and 7 days after cell seeding by confocal optical microscopy (Zeiss LSM 510/ConfoCor 2 system, Oberkochen, Germany). For all typologies, samples were fixed with 4% paraformaldehyde for 1 h and treated with 0.1% Triton X-100 to permeabilize cell membrane. Nuclei were stained with 4,6-Diamidino-2-phenylindole (DAPI) dye (1 μg/mL), actin filaments were stained with Phalloidin-AttoRho6G (100 μM), all by Sigma-Aldrich.
Phalloidin fluorescence was collected in a spectral window of 500 to 530 nm. For DAPI stain acquisition, 720 nm excitation wavelength and 450–500 nm spectral window emission were used. Several features characterizing cell morphology were identified by using the image analysis software ImageJ (NIH).

4.2.6 Statistics

The data are presented as mean ± standard deviation in triplicate. Characterization data and biochemical assays were analyzed using a by ANOVA followed by Bonferroni post-hoc test. $p < 0.05$ was considered a statistically significant value.

4.3 Results

At first, we tried to understand what the ideal concentration of nanoparticles was to be included in the two types of hydrogel material considered: collagen alone and the collagen / hyaluronic acid composite. We used three concentrations of SPIONs Fe_3O_4 already known in the literature [18] 50, 25, 10 μg/ml. Overall, for each different concentration of nanoparticles and hydrogel typology, a good cell viability and therefore biocompatibility of the materials was observed. (Fig 1) Differences in cell proliferation among different gel types were also observable: in detail, hydrogels enriched with hyaluronic acid showed the best results for every concentration here considered. (Fig1)
Furthermore, considering the percentage reduction data of alamar blue on average, the concentration of 25 μg / ml of NPs is the one that has been found to be more homogeneous over time with a peak at 5 days from cell seeding and a good seal both in collagen hydrogel than in the Col/HA composite. For this reason, it was decided to continue the study with the concentration of 25 μg / ml.

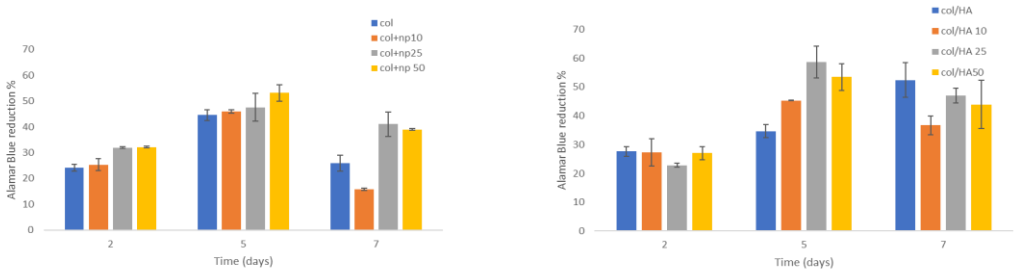

Figure 1. Percentage of Alamar blue reduction after 2,5,7 days of PDLSCs culture in each hydrogel (from left to right: Col and Col/HA) with three different concentrations of SPIONs (10, 25, 50 µg/ml).

Once the ideal concentration of nanoparticles to be inserted in the hydrogel had been established, we evaluated the trend over time of the proliferation of the periodontal ligament cells seeded both in the hydrogel of collagen only, with and without particles (Col-Col NP 25) then in the composite Col / HA with and without nanoparticles (Col/HA- Col/HA NP 25). (Fig 2a)

The same study was performed by subjecting the hydrogels to time-dependent magnetic stimulation (Col Mag, Col NP25 Mag, Col/HA Mag, Col/HA NP 25 Mag). (Fig 2b)

a

b

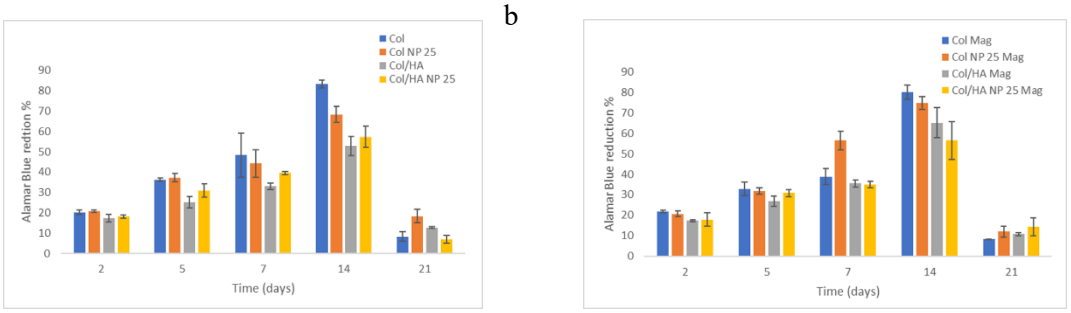

Figure 2. Cell proliferation of cell-laden hydrogels with and without magnetic nanoparticles NP (a) and with magnetic stimulation Mag (b) after 2, 5, 7 ,14, and 21 days of culture.

Fig 2b shows homogenous cell growth over time and consistent with the life cycle of cells even for hydrogels subjected to the action of the magnetic field, confirming the positive effect of magnetic stimulation on cell proliferation. In detail, we observe a growth peak at 14 days for all types of materials which leads to a condition of plateau and therefore a decrease in growth at 21 days from cell seeding.

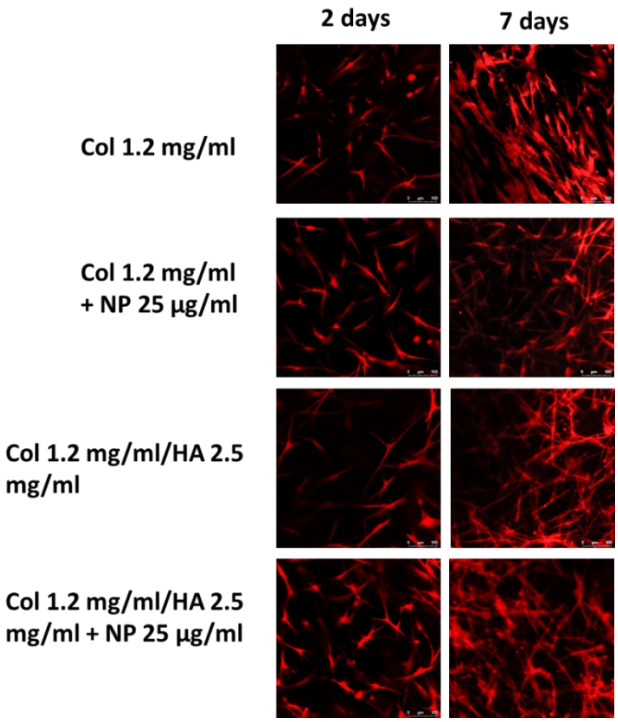

Figure 3. Fluorescence images of PDLSCs adhesion and morphology when cultured with Hydrogel of interest at 2 and 7 days after seeding. From top to bottom: Col 1.2 mg/ml; Col 1.2 mg/ml + NP 25 µg/ml; Col 1.2 mg/ml, HA 2.5 mg/ml; Col 1.2 mg/ml, HA 2.5 mg/ml + NP 25 µg/ml. F-actin of the PDLSCs is stained in red. Scale bar = 100 µm.

The cell proliferation data are highlighted by the images obtained with the confocal microscope analysis (fig 3) where through the staining of the actin filaments, performed with the use of rhodaminated phalloidin, it is possible to observe that after seven days from cell seeding in the formed by Collagen 1.2 mg / ml, hyaluronic acid at 2.5 mg / ml and nanoparticles at 25 µg / ml there is a more homogeneous and compact cellular distribution than other types of materials, underlining how the composite 3D structure is a better matrix than others.

Finally, being preliminary in vitro data for an *in vivo* bone regeneration study, we went to evaluate the ability of the various types of hydrogels to induce differentiation in the osteogenic sense in periodontal ligament cells. We then went to evaluate the ALP activity at 7,14,21 and 28 days after cell seeding in the hydrogel of collagen only, with and without particles (Col-Col NP 25) then in the composite Col / HA with and without nanoparticles (Col/HA- Col/HA NP 25). (Fig 4a)

The same study was carried out by stimulating the hydrogels with a time-dependent magnetic field. (Col Mag, Col NP25 Mag, Col/HA Mag, Col/HA NP 25 Mag). (Fig 4b)

a b

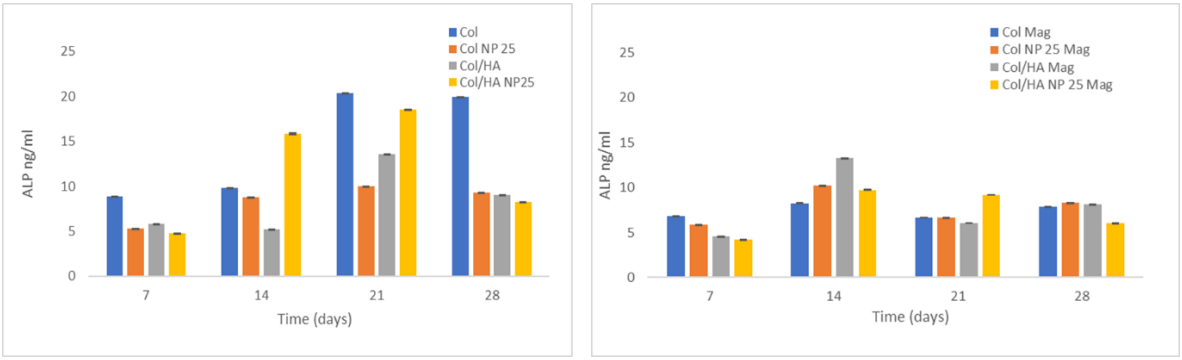

Figure 4. Alkaline phosphatase activity of cell-laden hydrogels with and without magnetic nanoparticles NP (a) and with magnetic stimulation Mag (b) after 7, 14, 21, and 28 days of culture.

The graphs show that hydrogels have a similar ability to allow PDLSCs cells to differentiate in an osteogenic sense both when they are not magnetically stimulated and when they are. In detail, the combination of the two materials, collagen and hyaluronic acid, combined with the presence of magnetic nanoparticles, seems to be the one that best responds to induction of differentiation. Although, more in-depth studies are needed in the future.

4.4 Discussion and Conclusion

Application of magnetic nanocomposite hydrogels for biomedical medicine offers a variety of possible cutting-edge scenarios. For example, by incorporating magnetic nanoparticles within hydrogel network, the nanocomposite network can remotely interact with external magnetic fields [19].

Accordingly, the aim of the project was the development of hydrogel injectable scaffolds with magnetic properties. Tested nanocomposite Hydrogels could be used for bone regeneration procedures and could represent an alternative to invasive surgery for bone defect treatment. In the next future step, the evaluation of scaffold's *in vivo* biocompatibility will be done on small animals (mice).

In particular, the injectable gel scaffolds will be tested in the mouse with a surgically induced mandibular bone defect to evaluate the potential of bone regeneration. To evaluate the influence of a magnetic field on the *in vivo* bone regeneration, a certain number of mice will be housed in magnetic cages specifically designed to recreate a magnetic field. The application of a time-dependent magnetic field will be considered as a magnetic stimulation. The magnetic field will be applied continuously for several hours (i.e., 6 h) a day and/or applied discontinuously considering several intervals (i.e., 18 minutes each). To evaluate the magnetic field effect there will be a control group that will not be magnetically stimulated. The magnetic experimental set-up will consist of a certain number of electromagnets and/or a solenoid equipped with specific electronic instruments. During the magnetic stimulation, samples will be placed at a specified distance to avoid the potential mutual influence. The proposed devices will be designed to possess specific morphological and architectural features together

with biological, mechanical, magnetic and mass transport properties which will be functional to bone tissue regeneration and to the control of bone remodeling during the orthodontic movement. Biological and biomechanical preliminary studies were performed as part of this future project. Design and preparation of injectable nanocomposite gel/hydrogel with magnetic features was achieved through the following steps: selecting the optimal nanocomposite Hydrogel matrix (Collagen /Hyaluronic acid) according to the *in vivo* tests, defining the magnetic nanoparticles (Fe_3O_4) concentration for the Hydrogel matrix, choosing the cellular line (PDLSCs periodontal ligament stem cells) and its optimal use density, studying the effect of magnetic field on the selected matrices with or without magnetic nanoparticles. In vitro tests of adhesion, proliferation and osteogenic differentiation on selected matrices were performed. The choice was taken due to rheological and mechanical properties of these matrices, according to the animal model (mice) established.

The optimal magnetic nanoparticles concentration and the cells proliferation were evaluated through a biological cell viability test (Alamar blue assay). Cell adhesion and spreading on the injectable nanocomposite hydrogel over time was showed though images confocal laser scanning microscopy images. The osteogenic differentiation rate of the PDLSCs laden into nanocomposites hydrogels was assessed by the Alkaline phosphatase test (ALP test). Tests were performed with or without a time dependent magnetic stimulation.

The preliminary in vitro results obtained show that the choice of the COL 1.2 mg/ml HA 2.5 mg/ml nanocomposite matrix, the PDLSCs cell line with a concentration of 60.000 cells/ml and the amount of magnetic nanoparticles (Fe_3O_4 25 µg/ml) seem to be a good starting point. The cells show excellent adhesion and proliferation in both magnetically non-stimulated and stimulated samples. Morphologically, the cellular distribution is homogeneous, thanks also to the presence of Fe_3O_4 magnetic nanoparticles. The material provides the favourable microenvironment capable to improve PDLSCs osteogenic differentiation. Moreover, the magnetic stimulation seems strengthen the biological response of the periodontal ligament stem cells, conforming the theories already present in literature; Huang et al. demonstrated that Fe_3O_4 nanoparticles could accelerate cell cycle progression [20]. As a result, the strengthened effect might affect ion channels on the cell membrane and initiate changes in cytoskeletal architecture under the magnetic field [21]. Thus, it is hypothesized that integration of electromagnetic field with the hydrogel could further control cell functions, such as adhesion, proliferation, and differentiation of stem cells. The nanocomposite magnetic Hydrogels synthesized in this study provides a promising scaffold for potential hard tissue engineering because the hydrogel responded to an external magnetic field, which could be used to direct the scaffold to the exact defect site remotely using an external magnet. Moreover, the hydrogel was cytocompatible with PDLSCs in vitro, as demonstrated by the increased PDLSCs viability when compared with the control. However, further cell studies with these gels in the presence of an external magnet are still needed to determine the combined effects of magnetic field and magnetic scaffold on cell functions. In addition, the presence of magnetic nanoparticles did not affect the viability of PDLSCs when compared with the control group. The in vitro results obtained will help to determine the parameters for *in vivo* experiments on animal models. In cell therapy, the fate of transplanted cells is the critical factor: few of the stem cells are able to survive [22] and their behavior is deeply affected by the microenvironment where they are forced to be cultured [23]. As 3D models for investigating

in vitro cell biology and physiology, alternative to 2D cell cultures, hydrogels have displayed offering to the cells a permissive template for their migration, proliferation and differentiation. [24]

4.5 References

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Chapter 5 Combination Design of Time-Dependent Magnetic Field and Magnetic Nanocomposites to Guide Cell Behavior

The concept of magnetic guidance is still challenging and has opened a wide range of perspectives in the field of tissue engineering. In this context, magnetic nanocomposites consisting of a poly(ε-caprolactone) (PCL) matrix and iron oxide (Fe_3O_4) nanoparticles were designed and manufactured for bone tissue engineering. The mechanical properties of PCL/Fe_3O_4 (80/20 w/w) nanocomposites were first assessed through small punch tests. The inclusion of Fe_3O_4 nanoparticles improved the punching properties as the values of peak load were higher than those obtained for the neat PCL without significantly affecting the work to failure. The effect of a time-dependent magnetic field on the adhesion, proliferation, and differentiation of human mesenchymal stem cells (hMSCs) was analyzed. The Alamar Blue assay, confocal laser scanning microscopy, and image analysis (i.e., shape factor) provided information on cell adhesion and viability over time, whereas the normalized alkaline phosphatase activity (ALP/DNA) demonstrated that the combination of a time-dependent field with magnetic nanocomposites (PCL/Fe_3O_4 Mag) influenced cell differentiation. Furthermore, in terms of extracellular signal-regulated kinase (ERK)1/2 phosphorylation, an insight into the role of the magnetic stimulation was reported, also demonstrating a strong effect due the combination of the magnetic field with PCL/Fe_3O_4 nanocomposites (PCL/Fe_3O_4 Mag).

5.1 Introduction

Tissue engineering and stem cell-based therapies are the most challenging fields in regenerative medicine. Tissue engineering is based on the synergistic combination of cells and appropriate scaffolds acting as functionally supportive biomolecules. A common scaffold is an interconnected porous structure that supports cell adhesion, proliferation and differentiation while promoting the extracellular matrix (ECM) analogue deposition that is necessary for tissue regeneration. Natural (e.g., alginate, collagen, chitosan, agarose, hyaluronic acid, and fibrin) and synthetic polymers (e.g., poly(ε-caprolactone)) have been employed to fabricate scaffolds for tissue engineering [1,2]. In this context, mechanical features are as important as hard tissues (e.g., bone), and they are stiffer (higher elastic modulus) and stronger (higher strength) compared to soft tissues [3–7]. If compared to other aliphatic polyesters, poly(ε-caprolactone) (PCL) has better viscoelastic and rheological properties, also resulting in it being easily workable and manipulatable [8]. Anyway, its mechanical properties are not suitable for high load-bearing applications (e.g., bone) [8,9]. Moreover, PCL hydrophobicity tends to inhibit cell adhesion and proliferation [9]. In regard to bone tissue engineering, different osteoinductive and osteogenic inorganic fillers (e.g., hydroxyapatite and bioactive glasses) are generally employed to enhance the biological and mechanical performances of PCL-based scaffolds [9]. Composite structures consisting of PCL reinforced with tricalcium phosphate (TCP) [8] or electrospun PCL nanofiber scaffolds with different amount of graphene oxide and graphene oxide surface grafted with poly(ethylene glycol) have been developed [9,10]. The inclusion of nanofillers in the PCL matrix up to a threshold concentration usually improves its mechanical properties [9]. A recent study also focused on the

development of PCL/layered double hydroxide (LDH) microsphere-aggregated nanocomposite scaffolds as suitable candidates for bone tissue engineering [11]. The results demonstrated that the inclusion of LDH nanoparticles improved the osteogenic differentiation and mechanical properties (e.g., compressive modulus) of mesenchymal stem cells. depending upon the LDH amount [11]. The grafting of carboxymethyl chitosan on electrospun PCL nanofibers was also proposed to manufacture scaffolds for bone tissue regeneration. The synergic effect of external stimulation factors (e.g., β-carotene and electromagnetic field) on the osteodifferentiation of adipose mesenchymal stem cells was analyzed [12]. Differently from conventional composites, nanocomposites based on a polymer matrix and inorganic-reinforcing nanofillers seem to better reproduce the natural structure of bone, a natural nanocomposite, and would represent a relevant candidate for bone tissue engineering. In addition, it has been widely demonstrated that nanocomposites induce a more efficient cell response and generally possess improved mechanical performance [1,7]. The use of magnetic nanoparticles in combination with an external magnetic field may represent an intriguing strategy to enhance bone tissue regeneration, as it has been reported to influence cellular metabolism. In the field of medicine, static and pulsed magnetic fields have been commonly used as components of the magnetic resonance technique, as well as to increase wound healing and to improve bone regeneration. Furthermore, recent data have also shown the mechanism of how a static magnetic field may influence biochemical properties using stem cells and different cell populations. The findings obtained to date suggest the potential use of therapies based on magnetic fields due to their easy application and possible effects on cells and organisms [13–19]. The concept of magnetic guidance has been extensively studied in the biomedical field (e.g., drug delivery, hyperthermia treatment for cancer, magnetic cell-seeding procedures, the stimulation of cell constructs, and the control of cell proliferation and differentiation) [19–32]. In this scenario, magnetic nanoparticles (MNPs) provide attractive possibilities as a direct consequence of their peculiar physical properties and their sizes. The application of an external magnetic field may also allow for the manipulation of their magnetic features, as well as the immobilization and/or transportation of the MNPs themselves and magnetic bioaggregates [33,34]. The potential to magnetically switch-on/switch-off a nanocomposite scaffold, consisting of a polymer matrix loaded with MNPs, could also be considered to deliver biomolecules (e.g., angiogenic factors) and stem cells, as well as to enhance cell adhesion, proliferation, and differentiation [17]. Even if many concerns remain about the long-term effects of iron-oxide-based phases such as maghemite (γ-Fe2O3) and magnetite (Fe_3O_4) [34–37] in the human body, over the past few years, the idea to design a fixed “station” whose magnetization can be switched on or off through the application or removal of external magnetic fields[14], respectively, has clearly led to the development of magnetic nanocomposite structures consisting of poly(ε-caprolactone) (PCL) loaded with Fe_3O_4 or iron-doped hydroxyapatite (FeHA) nanoparticles, as well as a challenging and programmed biofactor release [33,34,38]. Basically, the distribution of magnetic flux distribution can be altered by the presence of a magnetic scaffold, and magnetic field lines can be much more concentrated near and inside the scaffold. Magnetic gradients can be generated to allow the scaffold to attract and take up cells or bioagents bound to MNPs, which should function as transportation shuttles towards the magnetic scaffold [14]. The magnetic performances of both PCL/Fe_3O_4 and PCL/FeHA nanocomposites have already been analyzed, and

they showed a sigmoidal shape of the magnetization curve and a very low coercive field at 37◦C [34,38]. On the other hand, magnetic force could lead to changes in microenvironments, cell membranes, matrixes, cytoskeletons, and nucleoproteins. Therefore, signals can be transduced to the cell nucleus, modulating and promoting many biological responses. Several signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, are generally involved [39–41]. The expression of MAP protein kinases plays a fundamental regulatory role in cellular biology [41,42]. The phosphorylation of several substrate proteins, involving transcription factors, protein kinases and phosphatases, and further proteins, can be catalyzed by the activated MAP kinases. The MAPK pathway consists of a series of Ser/Thrkinases, including extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), p38, and ERK5 families, allowing for the regulation of the activity of specific transcription factors after the phosphorylation cascade. In regard to bone, tissue resorption and formation are related to the MAPK signaling pathway. To date, the effect of magnetic fields on osteogenic marker expression through different signaling pathways, including the protein kinase A (PKA) and MAPK, remains unclear [43–45]. Accordingly, the aim of the current research was to provide further insight into the combination design of a time-dependent magnetic field and PCL/Fe_3O_4 (80/20 w/w) nanocomposites for bone tissue engineering. The mechanical properties of PCL/Fe_3O_4 nanocomposites were first evaluated through small punch tests, whereas the combined effect of the magnetic stimulation and PCL/Fe_3O_4 nanocomposites on the behavior of human mesenchymal stem cells (hMSCs) was analyzed, with a focus on the MAPK signaling pathway.

5.2 Materials and Methods

5.2.1. Design and Manufacturing of PCL/Fe_3O_4 Nanocomposite Substrates

Nanocomposite pellets were first prepared according to a procedure already reported for the solution preparation and MNP dispersion [34,38,46]. Tetrahydrofuran (THF, Sigma-Aldrich, St. Louis, MO, USA) was used to dissolve poly(ε-caprolactone) (weight-average molecular weight Mw = 65,000, Aldrich,St. Louis,MO) pellets at room temperature. Polyvinylpyrrolidone (PVP)-coated Fe_3O_4 (99.5%, 25 nm, 0.2 wt % PVP, NanoAmor, Houston, TX) nanoparticles and, successively, ethanol were added to the polymer solution. A polymer/filler (PCL/Fe_3O_4) weight ratio (w/w) of 80/20 was employed. In regard to the nanoparticle dispersion, an ultrasonic bath (Branson 1510 MT, Danbury, CT) was also utilized. PCL/Fe_3O_4 (80/20 w/w) nanocomposite pellets were prepared from the obtained homogeneous paste after removing the solvent. The pellets were processed via a melting and molding technique to manufacture PCL and PCL/Fe_3O_4 (80/20 w/w) substrates. In brief, PCL or PCL/Fe_3O_4 pellets were heated to 100◦C, and the material was subsequently poured into a Teflon mold, allowing it to cool. A mylar strip was also placed on the top of the mold, and an appropriate load was distributed to achieve a flat surface for the specimens. In particular, disk-shaped specimens (diameter and thickness of 6.4 and 0.5 mm, respectively) were manufactured to perform small punch tests, whereas specimens with a larger diameter (10 mm) were employed for biological analyses.

5.2.2. Small Punch Test

Small punch tests were performed on PCL and PCL/Fe_3O_4 disk-shaped specimens (diameter of 6.4 and thickness of 0.5 mm) according to the ASTM F2183. Using a hemispherical head punch, disk-shaped specimens were loaded axisymmetrically inbending at a constant displacement rate of 0.5 mm/min until failure occurred. Load and displacement values were recorded during the test. The tests were carried out with an INSTRON 5566 testing machine (Norwood, MA, USA).

5.2.3. Scanning Electron Microscopy

The surface morphologies of the PCL and PCL/Fe_3O_4 substrates were analyzed with scanning electron microscopy (SEM) (FEI Quanta 200 FEG apparatus, The Netherlands).

5.2.4. Cell Culture

Human mesenchymal stem cells (hMSCs, Millipore, Germany), at the fourth passage, were cultured in Dulbecco's modified eagle medium (DMEM, Microtech, Italy) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM glutamine, and antibiotics (penicillin G sodium 100 U/mL, streptomycin 100 g/mL) at 37◦C and 5% CO_2. PCL and Fe_3O_4 substrates were prepared by soaking the structures in a solution of ethanol and antibiotics (penicillin/streptomycin), washed in phosphate-buffered saline (PBS, Sigma-Aldrich, Milan, Italy), and pre-wetted in FBS. hMSCs were seeded onto the substrates using a density of 1.0 x 10^4 cells/sample. After seeding, the cell-laden structures were incubated for 2 h (37 ◦C, 5% CO_2) and, successively, 1.5 mL of culture medium was added to each well in a 48-well plate.

5.2.5 Magnetic Stimulation

A time-dependent magnetic stimulation of the cell-laden constructs were performed at 1 day after cell seeding. Time-dependent means that the magnetic field varies as time (t) increases, and a sinusoidal wave is generally considered one of the commonly employed representation. Specifically, an external sinusoidal magnetic field was discontinuously applied for 6 h per day (20 intervals at 18 min each) benefiting from an optimized procedure [46]. Further cell-laden substrates were used as controls because they were placed in the same conditions without magnetic stimulation. A scheme of the experimental setup is shown in Figure 1.

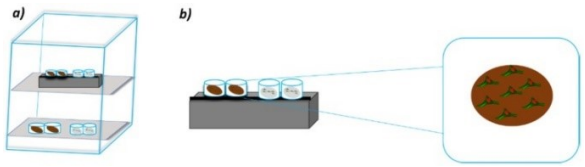

Figure 1. A scheme of the experimental setup that was employed for magnetic stimulation. (a) Incubator equipped with electromagnet; (b) further details related to cell-laden constructs and stimulation system.

The cell-laden constructs were exposed to a time-dependent magnetic field with an electromagnet placed below the wells. In order to avoid any kind of mutual influence, adequate technical solutions were adopted for the adjacent cell culture wells [46]. The test was performed three times in triplicate. As generally reported, the magnetically-stimulated PCL and PCL/Fe_3O_4 substrates were marked as PCL Mag and PCL/Fe_3O_4Mag, respectively, where "Mag" was used to indicate the application of the magnetic field.

5.2.6 Cell Metabolic Activity

The Alamar Blue assay (AbDSerotecLtd., UK) was used to assess cell viability and proliferation. At 1,4,7,14, and 21 days after cell seeding, the cell-laden substrates were rinsed with PBS (Sigma–Aldrich, Italy) and 200 µL of DMEM without phenol red (HyClone, UK) containing 10% (v/v) Alamar Blue was added for each sample. The samples were incubated in 5% CO_2 diluted atmosphere for 4 h at 37◦C. After removing one hundred microliters of the solution, it was transferred to the well plate. A spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland) was utilized, and the optical density was measured at wavelengths of 570 and 595 nm. The experiments were done at least three times in triplicate.

5.2.7. Alkaline Phosphatase Activity

Samples were removed from the medium and washed twice with PBS at days 3, 7, and 14. The cell-laden substrates were then incubated in 1 mL of a lysis buffer and centrifugated. A cell density of 1×104 cells/sample was employed. The alkaline phosphatase (ALP) activity was measured with an enzymatic assay (SensoLyte pNPP alkaline phosphatase assay kit - AnaSpec Inc., Fremont, CA, USA), which was based on the p-nitrophenyl phosphate (pNPP). Normalized ALP activity (ALP/DNA) was calculated by dividing the ALP activity over the DNA content with the Quant-iT PicoGreen assay kit (Molecular Probes Inc., Eugene, OR, USA), which allows for the detection and quantification of DNA. According to the manufacturer's protocol, the working solutions were prepared and the procedure was followed.

5.2.8. Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) was also employed as an optical imaging technique for the analysis of cell adhesion and spreading at 4, 7, and 14 days after seeding, using a Zeiss LSM 510/ConfoCor 2 system (Oberkochen, Germany) equipped with argon and helium–neon lasers and with a 10X objective. In brief, rhodamine phalloidin staining was performed, constructs were imaged, and actin filaments were visualized. CLSM images were successively analyzed with Image J software (NIH, Bethesda, MD, USA) to determine the cell morphology [2,46,47]. The cell shape factor (Φ) was calculated as follows:

$$\Phi = \frac{4\pi A}{p2} \quad (1)$$

where A and P represent the area and the perimeter of a cell, respectively. The greatest area-to-perimeter ratio is obtained for circular objects, and a perfect circle has a shape factor of 1. Conversely, a thin thread-like object shows the lowest shape factor that approaches zero [2,46,47].

5.2.9. Immunoblot Analysis

The following primary antibodies were used: rabbit phosphor ERK(Thr202/Tyr204) (CellSignaling) and rabbit ERK1/2 (Santa Cruz). Polyclonal antibodies were employed at working dilutions of 1:1000. Antibody protein complexes were detected by horseradish peroxidase (HRP)-conjugated antibodies and an enhanced chemiluminescent (ECL) system (both from Amersham Pharmacia, Piscataway Township, NJ, USA). PCL and PCL/Fe_3O_4 substrates were first prepared by soaking the structures in a solution of ethanol and antibiotics (penicillin/streptomycin), washed in PBS (Sigma-Aldrich, Italy) and pre-wetted in FBS. hMSCs (density of 1.8 × 105 cells/sample) were seeded onto the substrates (diameter and thickness of 34 and 0.5 mm, respectively) in a 6-well plate. After seeding, the cell-laden structures were incubated for 2 h (37◦C, 5% CO2) and, successively, 1.5 mL of a culture medium was added to each well. Four days after magnetic stimulation (6 h per day for 20 intervals of 18 min each), cell-laden substrates were serum-deprived overnight and left untreated or treated with epidermal growth factor (EGF—100 ng/mL; treatment times of 15 and 30 min). The cells were collected with trypsin and then centrifuged, and the obtained pellets were stored at −80◦ C. hMSCs were lysed in a saline buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl (pH 7.5), 70 mM NaCl, and 1% Triton. The lysates were cleared by centrifugation at 15,000 x g for 10min and quantified by the Bradford method. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on cell lysates, and the proteins were transferred to a nitrocellulose membrane for 3h. Filters were blocked for 1h at room temperature in Tween-20 Tris-buffered saline (TTBS) (TBS-Sigma, 0.1% Tween 20, pH 7.4) with 5% w/v non-fat dry milk. Blots were then incubated with a primary antibody (p-ERK; ERK1/2) overnight. They were washed three times with a TTBS buffer and incubated for 1h with a secondary antibody (peroxidase-coupled anti-rabbit) in TTBS-5% (w/v) non-fat dry milk. Reactive signals were detected by an ECL Western blotting analysis system. The autoradiography images were acquired and analyzed with Image-J software (NIH, Bethesda, MD, USA). Thus, cell-laden substrates were treated with EGF at different time points (15 and 30 min) in the absence or presence of a time-dependent magnetic field applied for 6 h per day (20 intervals of 18 min each) for 4 days. The magnetically-stimulated cell-laden substrates were compared to the control groups (unstimulated polymeric and nanocomposite substrates) in the presence and absence of EGF.

5.2.10. Statistical Analysis

Data are reported as mean value ± standard deviation and analyzed by ANOVA followed by Bonferroni post hoc test. A value of $p < 0.05$ was considered statistically significant.

5.3. Results

5.3.1. Small Punch Test

Load-displacement curves from small punch tests on disk-shaped specimens (PCL and PCL/Fe_3O_4) displayed an initial linear trend, a subsequent decreasing slope, a maximum load, and, then, a final decrease of the load until failure occurred. The initial local maximum (peak load) and the area under the load-displacement curve (work to failure) were assessed (Table 1).

Table 1. Results from small punch tests: peak load and work to failure.

Materials	Peak Load (N)	Work to Failure (mJ)
PCL	28.1 ± 2.1	31.4 ± 4.2
PCL/Fe_3O_4 (80/20 w/w)	34.4 ± 2.6	30.1 ± 5.3

PCL/Fe_3O_4 substrates provided values of peak load that were significantly higher than those achieved for PCL ($p < 0.05$). In terms of work to failure, no statistically significant differences were found between the two groups ($p > 0.05$).

5.3.2. Scanning Electron Microscopy

SEM images of polymeric (PCL) and nanocomposite (PCL/Fe_3O_4 80/20 w/w) substrates are reported in Figure 2. The surface topography of the nanocomposites was clearly influenced by the presence of aggregates and MNPs distributed in the matrix.

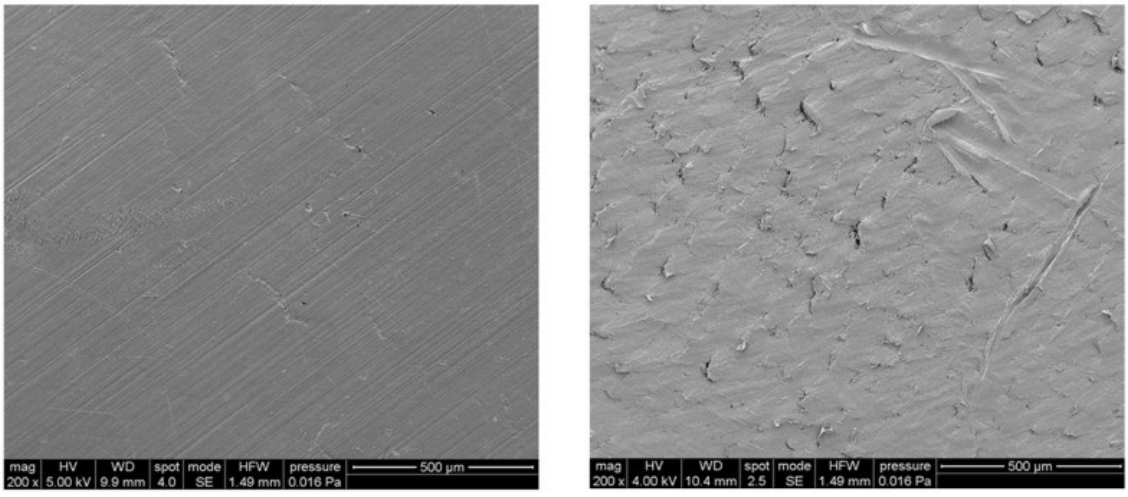

Figure 2. Typical SEM images of poly(ε-caprolactone) (PCL) (left) and PCL/Fe_3O_4 (right) substrates. Scale bar: 500 µm.

5.3.3. Cell Metabolic Activity

Cell viability and proliferation were analyzed for the different kinds of substrates, and the results are reported as a percentage of Alamar Blue reduction (Figure 3).

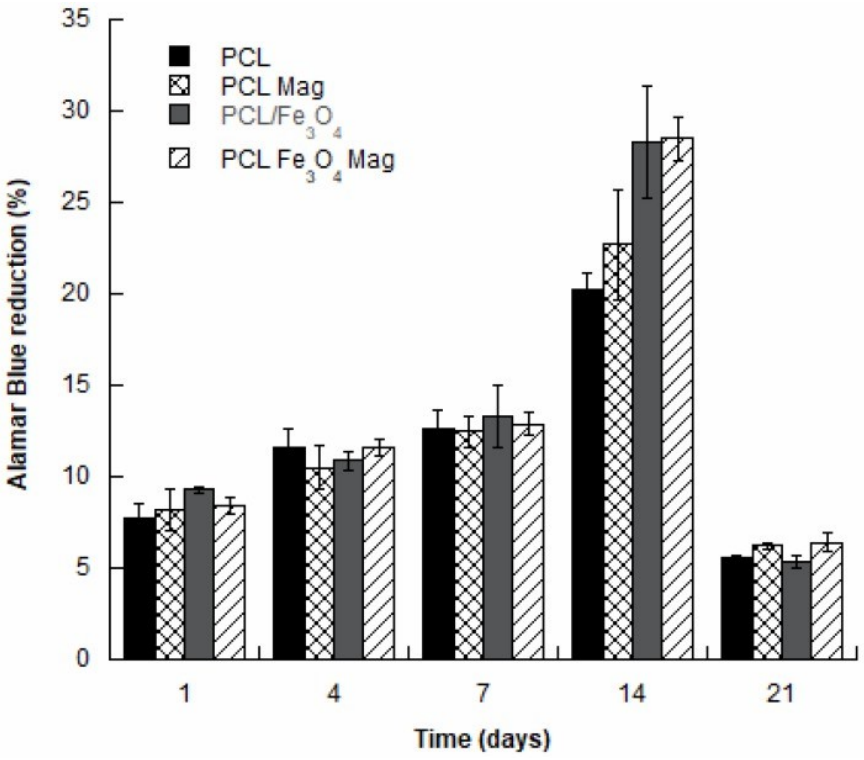

Figure 3. Percentage of Alamar Blue reduction evaluated for PCL, PCL/Fe_3O_4, PCL Mag and PCL/Fe_3O_4 Mag at different time points.

The results evidenced that all samples supported the adhesion and proliferation of hMSCs. As reported, the number of viable cells was associated with the magnitude of dye reduction. A significant increase ($p < 0.05$) of Alamar Blue reduction was evident over time, suggesting that hMSCs can survive and proliferate. One day after seeding, the results indicated that hMSCs were viable on both PCL and PCL/Fe_3O_4 substrates, with and without magnetic stimulation. An increase in the value of percentage of Alamar blue reduction was evident up to 14 days after seeding. Even through no statistically significant differences ($p > 0.05$) were observed among the different groups at 1, 4, and 7 days, at day 14, the percentage of Alamar Blue reduction was significantly higher ($p < 0.05$) for PCL/Fe_3O_4 and PCL/Fe_3O_4 Mag if compared to the PCL substrates. However, no statistically significant differences were found between the PCL/Fe_3O_4 and PCL/Fe_3O_4 Mag substrates ($p > 0.05$). In addition, at 21 days after cell seeding, a significant decrease ($p < 0.05$) in the percentage of Alamar Blue reduction was found for the different kinds of cell-laden constructs (Figure 3).

5.3.4. Alkaline Phosphatase Activity

The alkaline phosphatase activity was measured at 3, 7, and 14 days for each group (PCL, PCL/Fe_3O_4, PCL Mag, and PCL/Fe_3O_4 Mag) and normalized to DNA content (ALP/DNA) to quantitatively assess early osteogenic differentiation. Figure 4 reports some differences in terms of results, as the ALP/DNA ratio peaked at 7 days in the case of PCL, PCL Mag and PCL/Fe_3O_4, whereas the values significantly increased ($p < 0.05$) over the analyzed time period (from 3 to 14 days) for PCL/Fe_3O_4 Mag.

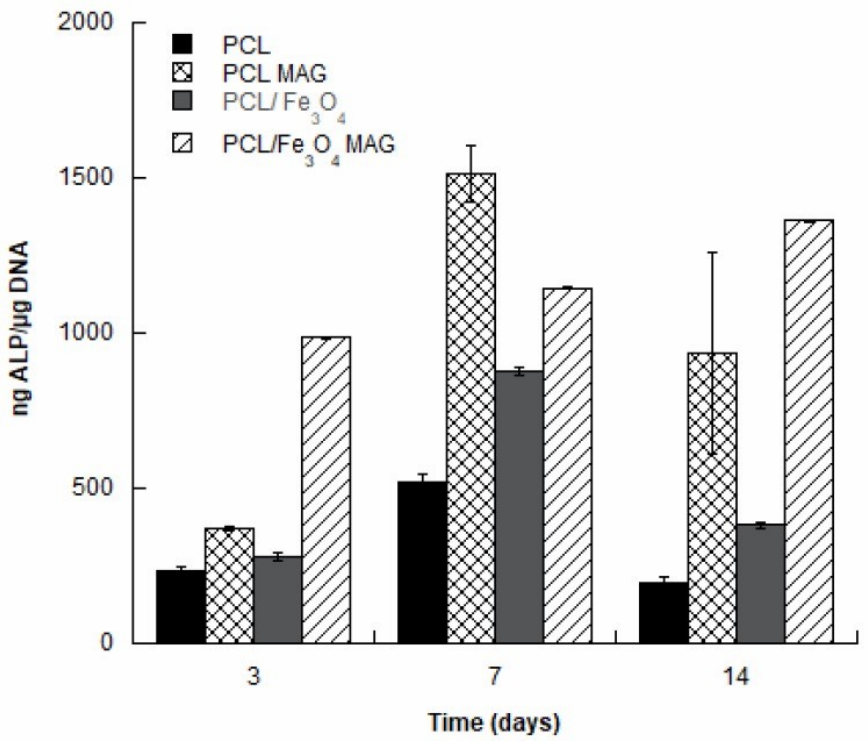

Figure 4. Normalized alkaline phosphatase (ALP) activity (ALP/DNA) for PCL, PCL/Fe_3O_4, PCL Mag, and PCL/Fe_3O_4 Mag at 3, 7, and 14 days after cell seeding.

In the case of both PCL Mag and PCL/Fe_3O_4, significantly higher values were found in comparison to PCL ($p < 0.05$). However, at different time points, the unstimulated PCL/Fe_3O_4 showed a lower ALP activity compared to the magnetically-stimulated PCL (PCL Mag). The observed differences were statistically significant ($p < 0.05$). On the other hand, the effect of the magnetic stimulation on PCL/Fe_3O_4 led to the highest ALP activity at 3 days, as well as an increasing trend over time (see PCL/Fe_3O_4 Mag).

5.3.5. Confocal Laser Scanning Microscopy

CLSM performed on all the cell-laden substrates provided qualitative results in terms of cell adhesion and spreading at 4, 7, and 14 days after seeding. The CLSM images confirmed the results obtained from the Alamar Blue assay. The number of viable cells on the nanocomposite substrates under magnetic stimulation strongly increased over time. Cell morphology also varied over time, changing from a geometry characterized

by few ramifications to a thread-like geometry with an increased number of ramifications (Figure 5), thus suggesting the establishment of a higher number of cell–cell and cell–material interactions.

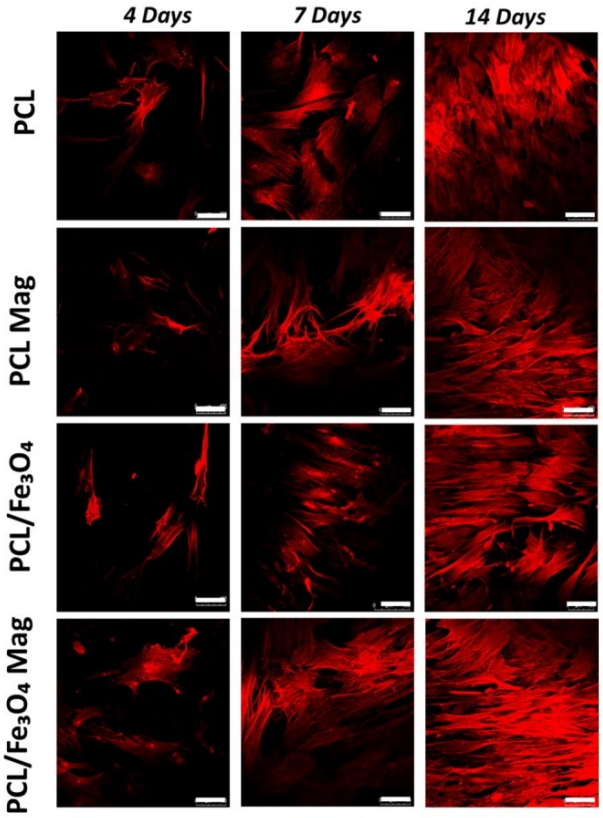

Figure 5. Confocal laser scanning microscopy (CLSM) analysis on cell-laden substrates at different time points. Images of rhodamine phalloidin-stained actin filaments(red). (left column) From top to bottom: PCL, PCL Mag, PCL/Fe_3O_4, and PCL/Fe_3O_4 Mag at 4 days. (middle column) From top to bottom: PCL, PCL Mag, PCL/Fe_3O_4 80/20, and PCL/Fe_3O_4 80/20 Mag at 7 days. (right column) From top to bottom: PCL, PCL Mag, PCL/Fe_3O_4 80/20, and PCL/Fe_3O_4 80/20 Mag at 14 days. Scale bar: 100 μm.

Further studies of cell adhesion and spreading were carried out based on CLSM images with the aim of determining the shape factor of the cells. Figure 6 reports the shape factor at 4, 7, and 14 days. As an example, in the case of PCL/Fe_3O_4 nanocomposites under magnetic stimulation (PCL/Fe_3O_4 Mag), the values of the cell shape factor significantly decreased ($p < 0.05$) from 0.23±0.02 at day 4 to 0.06±0.01 at day 14. Even though no statistically significant differences ($p > 0.05$) were observed among the several groups in terms of cell shape factor at day 4, significantly lower values ($p < 0.05$) were found in the case of PCL/Fe_3O_4 Mag at 7 and 14 days. Furthermore, no significant differences ($p > 0.05$) were found among PCL, PCL Mag, and PCL/Fe_3O_4 at 7 and 14 days.

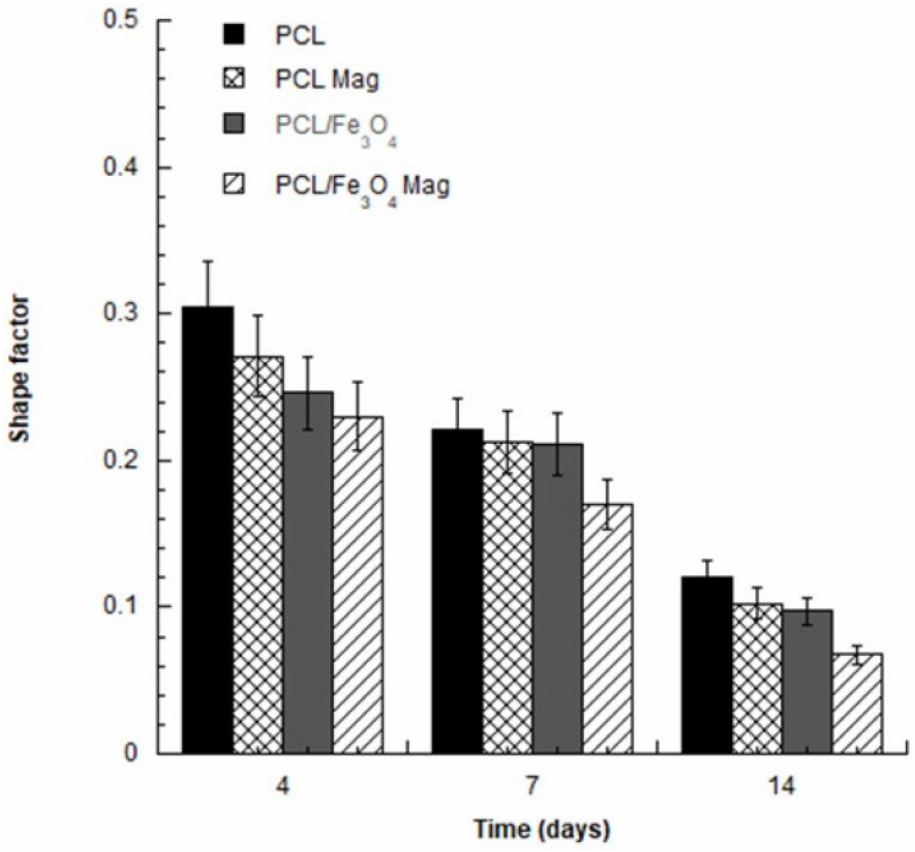

Figure 6. Shape factor evaluated from the CLSM images of human mesenchymal stem cells (hMSCs) on PCL and PCL/Fe_3O_4 substrates (with or without magnetic stimulation) at different time point.

5.3.6. Immunoblot Analysis

The p-ERK1/2 expression was investigated for the different kinds of cell-laden substrates. Specifically, the phosphorylation levels of ERK1/2 were monitored by Western blotting analysis. In regard to both PCL and PCL/Fe_3O_4, the presence of EGF led to phosphorylation levels of ERK1/2 that were generally higher than those obtained in the absence of EGF, also increasing with the EGF treatment time (Figure 7). Moreover, the magnetic stimulation further improved the phosphorylation levels of ERK1/2, also providing interesting results in the absence of EGF.

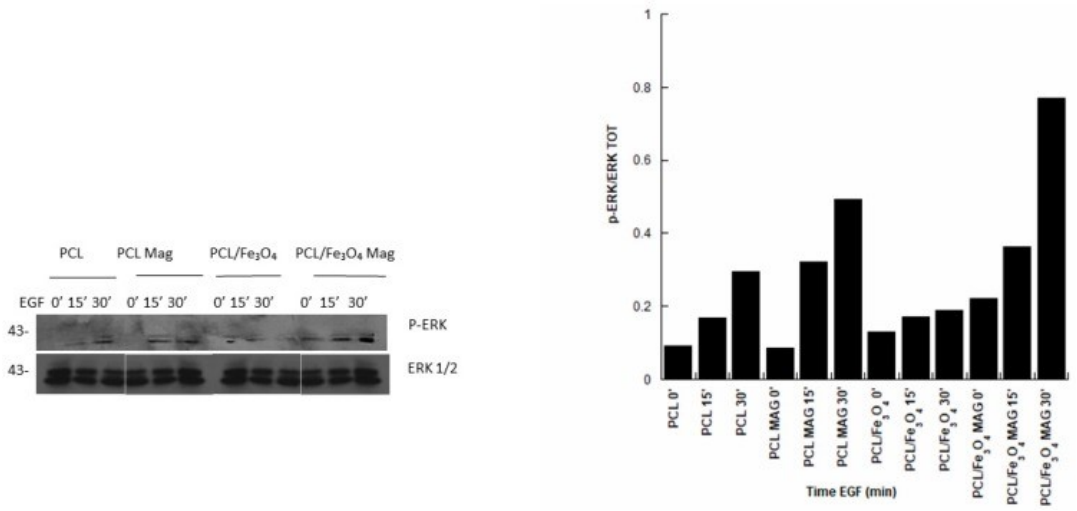

Figure 7. Effect of magnetic field on extracellular signal-regulated kinase (ERK)1/2 phosphorylation. Western blotting analyses were performed on substrates stimulated with a time-dependent magnetic field or left unstimulated. The experimental groups were treated with epidermal growth factor (EGF) at indicated time points (15 and 30 min). Cell-laden substrates were then lysed and subjected to immunoblot analysis with the indicated antibodies. PCL0',PCL/Fe_3O_4 0',PCLMag0',and PCL/Fe_3O_4 Mag 0' indicate cell constructs in the absence of EGF.

However, the highest levels were found for PCL/Fe_3O_4 Mag (Figure 7). In Figure 7, samples marked as PCL 0', PCL/Fe_3O_4 0', PCL Mag 0', and PCL/Fe_3O_4 Mag 0' represent cell constructs in the absence of EGF.

5.4. Discussion

The concept of magnetic guidance may be properly considered to design devices where many features related to the magneto-mechanical activation/stimulation of cell-laden constructs, magnetic cell-seeding techniques, and tailored cell proliferation and differentiation—as well as to the release of biomolecules or bioactive factors that can be linked to magnetic nanocarriers—can be simultaneously integrated [14–32,38,47,48]. The possibility to design magnetic nanocomposite substrates represents a great challenge in the field of bone tissue regeneration. The rationale should be the development of magnetic nanocomposite structures that may be magnetized in situ by applying an external magnetic field with the aim to control specific cellular processes. In regard to the preparation of polymer-based nanocomposites, over the past few years, ultrasonication has been considered an efficient methodology to enhance the dispersion of nanofillers in a polymer solution, allowing for the excitation of the resonance vibrations of nanofiller clusters and/or to break up them [49]. Further works on the development of PCL/MNP scaffolds have frequently reported the use of ultrasonication as a strategy to homogeneously disperse MNPs (e.g., magnetite and iron oxide-based nanoparticles) in different concentrations with respect to the total PCL mass [19,50]. In the current research, a procedure that had already been reported for the preparation of the PCL solution and MNPs (e.g., PVP-coated Fe_3O_4 and iron-doped hydroxyapatite) dispersion under ultrasonication [34,38,46] was adopted to prepare PCL/Fe_3O_4 pellets for the manufacturing of nanocomposites. Moreover, many studies have dealt with the high dispersion stability of PVP-coated iron-oxide nanoparticles in different solvents, so no chemical methods, and sonication methods in

the presence of PVP as stabilizer to produce well-dispersed nanoparticles [51,52]. As frequently reported, in polymer nanocomposites, the different ductility values between the inorganic nanofillers and the polymeric matrix generally cause stress concentration and discontinuities in the stress transfer mechanism at the nanoparticle/matrix interface [46,48]. According to previously reported data, a decrease of the functional properties may be observed beyond a threshold concentration of nanoparticles, as they can act as "weak points" instead of a reinforcement for the polymeric matrix, thus weakening structures [46,48,53]. Additionally, magnetization measurements had already been performed on PCL/Fe_3O_4 nanocomposites, and the results provided information in terms of a very low coercive field and saturation magnetization levels [34]. As for some physical, magnetic, and functional characteristics of the employed PCL/Fe_3O_4 nanoparticles (99.5%, 25 nm, 0.2 wt % PVP, NanoAmor, Houston, TX), previous studies [34,54] on the development of additively manufactured PCL/Fe_3O_4 scaffolds have demonstrated that the inclusion of these MNPs led to magnetization curves with saturation magnetization values ranging from 4 to 6 emu/g, depending upon the amount of MNPs. Starting from magnetization curves for the PCL/Fe_3O_4 scaffolds and fitting of the experimental data with the Langevin function, a particle diameter of 28 nm was estimated [34]. In this scenario, the current investigation provided a further insight into the combination design of a time-dependent magnetic field and PCL/Fe_3O_4 (80/20 w/w) nanocomposites to potentially guide cell behavior. The mechanical properties of PCL/Fe_3O_4 nanocomposites developed using melting and molding techniques were evaluated through the small punch test, which is a reproducible miniature specimen test method. Correlations between mechanical properties that were evaluated through uniaxial tensile tests and small punch tests were found for some low-alloy steels, providing analytical formulations that properly described the uniaxial stress–strain behavior [55]. This test method had already been employed for the evaluation of the mechanical properties of ultra-high molecular weight polyethylene employed in surgical implants and retrieved acrylic bone cements, as well as of PCL loaded with organic–inorganic hybrid fillers and PCL/iron-doped hydroxyapatite nanocomposite substrates [6,38]. It has been well documented that the data obtained from small punch tests do not provide information on yield stress and Young's modulus, especially in the case of polymer-based nanocomposites, as the loading configuration leads to lateral bending and large biaxial deformations [56]. Taking into account a small deformation region and the initial slope of the obtained curves, even though finite element analysis can be performed to assess mechanical properties such as the Young's modulus, this approachis sensitive to some parameters including the further mechanical characteristics of the specimen [56]. Accordingly, the addition of nanofillers influence the elastic and post-yield behaviour and the neglecting of these features most of ten leads to an incorrecte valuation of the modulus. Thus, it would be complex to separately assess these properties using a single test configuration [56]. For this reason, in the current research, relative mechanical properties were reported as punching properties that were evaluated by small punch test while allowing for a comparison between the PCL and PCL/Fe_3O_4 substrates. The peak load and the work to failure were determined as a relative measure of the strength and the ability of the material to absorb energy before breaking (i.e., toughness), respectively. The inclusion of Fe_3O_4 nanoparticles improved the punching properties, as the values of peak load achieved for PCL/Fe_3O_4 nanocomposites was significantly higher ($p < 0.05$) than those obtained for PCL without negatively

affecting the work to failure($p>0.05$)(Table1), even if SEM images showed a surface topography where the presence of aggregates was evident. Though the results summarized in Table 1 (peak load and work to failure) do not yield functional information in terms of mechanical properties for bone tissue engineering applications, it is worth noting that previous works [34,54] on the design of additively manufactured PCL/Fe_3O_4 nanocomposite scaffolds have preliminarily demonstrated the potential to match the strength and modulus of the human cancellous bone (4–12 MPa and 0.02–0.5 GPa, respectively [47,57]) by tailoring composition, architectural features (e.g., lay-down pattern), pore shape and size. However, Alamar Blue assay, normalized ALP activity (ALP/DNA), CLSM analysis, cell shape factor, and p-ERK1/2 expression provided interesting and, in many cases, unreported information, also suggesting how the synergistic combination of PCL/Fe_3O_4 nanocomposites with a discontinuous application of an external magnetic field (6 h per day with 20 intervals of 18 min each) may impact cell behavior. The discontinuous magnetic field would seem to significantly influence the behavior of hMSCs in terms of cell viability and differentiation. Even though an improvement of cell viability should be probably ascribed to the effect of magnetic stimulation ,surface topography, and chemistry(Figure3),as higher values of the percentage reduction of Alamar Blue were found in the case of PCL/Fe_3O_4 and PCL/Fe_3O_4 Mag at day 14, the results in terms of normalized ALP activity evidenced important differences affecting the cell differentiation process (Figure 4). The effect of the material alone and in combination with the magnetic field was analyzed. In the absence of magnetic stimulation, the effect of surface topography and chemistry on cell osteogenic differentiation was clearly evident, as PCL/Fe_3O_4 nanocomposite showed a level of ALP activity that was higher than that found for PCL (Figure 4). For each material (PCL or PCL/Fe_3O_4), the application of a time-dependent magnetic field provided a higher level of ALP activity for the magnetically-stimulated substrates (PCL Mag or PCL/Fe_3O_4 Mag), if compared to the unstimulated ones (PCL or PCL/Fe_3O_4). In addition, for each material (PCL or PCL/Fe_3O_4), the applied time-dependent magnetic field differently influenced the behavior of hMSCs over time, since the ALP/DNA ratio peaked at 7 days for PCL Mag, as with all the unstimulated substrates, whereas the magnetic stimulation led to increasing levels of ALP activity in the case of PCL/Fe_3O_4 (see PCL/Fe_3O_4 Mag in Figure 4), also resulting in a prolonged differentiation. The obtained results suggested that the combination of PCL/Fe_3O_4 nanocomposites with a time dependent magnetic field (PCL/Fe_3O_4Mag) was able to provide and improve the long-term maintenance of hMSC differentiation, which was more rapidly lost in the case of both magnetically-stimulated PCL (PCL Mag) and unstimulated substrates (PCL and PCL/Fe_3O_4). CLSM images and cell shape factor allowed us to further analyze cell adhesion and spreading at different time points, indicating a correlation between the reduction in cell shape factor over time with the establishment of multiple cellular extensions and, hence, an increase in total cell area. This may be directly ascribed to an enhancement of cell adhesion and spreading. The differentiation process is strongly related to the degree of cell spreading [58]. In particular, an increased cell spreading would improve the osteogenic differentiation of hMSCs due to a potential enhancement of the cytoskeletal contractility that favors osteogenesis [58]. Comparing the different cell-laden substrates at 7 and 14 days, the lower values of the cell shape factor for the magnetically-stimulated PCL/Fe_3O_4 nanocomposites were consistent with the results from the normalized ALP activity, justifying the differences found in the

differentiation process. Anyway, osteogenic differentiation is a complex process that involves several biophysical cues and biological factors [59]. Even if cell spreading, which was measured through shape factor, plays a crucial role in the regulation of these process, many concerns remain on how it may influence the maintenance of the committed phenotype after MSC differentiation [59]. On the other hand, the phosphorylation levels of ERK1/2 also evidenced the role of the material–magnetic field combination. Specifically, the possibility to activate the MAPK pathway was demonstrated for both magnetically-stimulated and unstimulated PCL and PCL/Fe_3O_4 substrates in the presence of EGF (Figure 7). The magnetic stimulation positively influenced the EGF-dependent phosphorylation of ERK and also provided interesting results in the absence of EGF, especially for PCL/Fe_3O_4 substrates (Figure 7). The application of a time-dependent magnetic field improved the activation of the MAPK pathway, as evidenced by an increase of the ERK phosphorylation for both PCL and PCL/Fe_3O_4. It is also worth noting how the best results in terms of ERK phosphorylation were achieved by combining magnetic stimulation and magnetic nanocomposites (PCL/Fe_3O_4 Mag), thus stressing the important role of the material–magnetic field combination (Figure 7). Accordingly, the obtained findings would seem to corroborate the role of magnetic stimulation. Strategies to improve bone tissue regeneration, involving magnetic nanocomposite scaffolds with or without the application of a magnetic field, have been widely reported in the literature [42]. ALP activity, the mRNA expression of specific markers (e.g., osteocalcin and osteopontin) and alizarin red staining on nanocomposite scaffolds consisting of PCL and magnetite nanoparticles showed how magnetic features can stimulate the differentiation process [60]. The potential to upregulate specific integrin subunits and to activate downstream pathways (e.g., focal adhesion kinase-FAK, paxillin, p38, and ERK/MAPK) was reported [60]. Furthermore, runt-related transcription factor 2 (Runx2) has an important role in promoting bone regeneration, and several factors that are involved in osteogenic differentiation regulate its expression [61,62]. Runx2 phosphorylation is also mainly mediated by ERK1/2 [62]. The combination of a static magnetic field with magnetic PCL/Fe_3O_4 scaffolds provided interesting information in terms of ALP activity as well as of the expression of bone-associated genes (e.g., Runx2 and Osterix). The role of such a combination further evidenced the possibility to activate integrin signaling pathways (e.g., FAK, paxillin, and Ras homolog family member A-RhoA), as well as to upregulate bone morphogenetic protein-2 and the phosphorylation of Smad 1/5/8 [63].

For this reason, the current study may be considered as a first step toward are search involving the combination design of 3D additive manufactured magnetic scaffolds and a time-dependent magnetic field, with the aim to provide a prompt on the assessment of the behavior of hMSCs through further analyses on osteogenic differentiation, involving alizarin red staining and the gene expression of osteogenic markers such as bone morphogenetic protein-2 (BMP-2), Runx2, and collagen type 1 alpha 1 (COL1A1), and to study the MAPK pathway alterations related to magnetic stimulation in cell-laden constructs.

5.5. Conclusions

Within the limitations of the proposed research, the following conclusions were reached:

1. The possibility to develop PCL/Fe_3O_4 (80/20w/w) substrates with improved mechanical properties (higher strength than PCL without negatively affecting the work to failure) was demonstrated by the small punch test.

2. The combination of a time-dependent magnetic field with PCL/Fe_3O_4 nanocomposites (PCL/Fe_3O_4 Mag) impacted the behavior of hMSCs, especially resulting in a prolonged cell differentiation.

3. The effect of a time-dependent magnetic field in increasing ERK phosphorylation levels and, hence, in the activation of the MAPK pathway, was reported, also corroborating previous findings on the combination of a magnetic field and magnetic nanocomposite structures.

4. The role of the material–magnetic field combination was revealed, as the highest ERK phosphorylation levels were found in the case of PCL/Fe_3O_4 Mag.

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Chapter 6 Role of magnetic features in additive manufactured scaffolds for enhanced bone tissue.

6.1 Introduction

The concept of magnetic guidance has opened a wide range of perspectives in the field of tissue regeneration. Accordingly, the aim of the current research was to design magnetic responsive scaffolds for enhanced bone tissue regeneration. Specifically, magnetic nanocomposite scaffolds were additively manufactured using 3D fibre deposition technique. The mechanical and magnetic properties of the fabricated scaffolds were first assessed. The role of magnetic features on the biological performances was properly analyzed.

Many studies have been already focused on artificial bone grafting for bone defect repair even if there are still some concerns related to delayed union or nonunion as a consequence of the loss of cell viability.[1–3] The incorporation of growth factors into scaffolds represents a common method for improving cell viability.[1,4] However, a great limit is related to the short half-life of growth factors which results in the rapid loss of their functions.[1] In this context, magnetic field stimulation may activate many sensitive receptors on cell surface, further stimulating signaling pathways to improve cell activity.[1] Moreover, with regard to a bone defect, the application of a continuous magnetic field may provide a sustained boost to improve cell activity. In addition, the scaffold-host bone integration, the increase of calcium content and newly formed bone density can be promoted by the magnetic field stimulation. This clearly accelerates the bone healing process.[1] It is frequently reported that magnetic nanoparticles (MNPs) with size lower than 30 nm show a superparamagnetic behavior. Thus, each particle can be considered as a single magnetic domain.[1] These considerations have allowed to speculate that the presence of MNPs embedded in the polymer matrix of the scaffolds should create a nanoscale magnetic field, allowing to construct microenvironments within the fabricated scaffolds, which are able to produce micro-magnetic driving force at the scaffold-cell interface. Contextually, the activation of many sensitive receptors on cell surface can be possible, improving cell activity and promoting bone formation. Fe_3O_4 MNPs show interesting magnetic properties and possess excellent biocompatibility and nontoxicity. For this reason, they have been widely investigated in the biomedical field (e.g., magnetic targeting, hyperthermia, tissue engineering).[1] Fe_3O_4 MNPs have also been approved by US Food and Drug Administration for clinical use.[1] The presence of Fe_3O_4 MNPs can obviously improve the mechanical properties of polymer scaffolds. Taking into account the advantages of Fe_3O_4 MNPs, they have been incorporated into biomaterials for tissue engineering applications.[1] For example, biocompatible Fe_3O_4/chitosan scaffolds with high magnetism were developed,[1] whereas magnetic nanocomposites and 3D additive manufactured scaffolds consisting of poly(ε-caprolactone) (PCL) loaded with Fe_3O_4 or iron-doped hydroxyapatite (FeHA) nanoparticles were designed, fabricated and analyzed.[5–8] The aim of the current study was to further analyze the role of magnetic features in additive manufactured PCL/Fe_3O_4 scaffolds for enhanced bone tissue regeneration.

6.2 Materials and Methods

PCL/Fe_3O_4 nanocomposite pellets (10% w/w of Fe_3O_4) were first prepared according to an already reported procedure.[6] Cylindrical scaffolds were additively manufactured using 3D fiber deposition technique. A needle with an inner diameter of 400 μm was used to extrude/inject the material at a temperature of 130°C. The nanocomposite fibers were deposited according to the selected lay-down pattern (i.e., 0°/0°/90°/90°). The fiber spacing (i.e., center-to-center distance) was set to 900 μm. A nitrogen pressure of 8.0 bar and a deposition speed of 35 mm min−1 were employed. Mechanical compression tests were performed on the fabricated 3D PCL/Fe_3O_4 scaffolds. The scaffolds were tested at a cross-head speed of 1 mm min−1 up to a strain of 0.4 mm mm−1, using an INSTRON 5566 testing system. The "apparent" stress and strain were calculated as described by De Santis et al. [6] The slope of the initial linear portion of the stressstrain curve was considered to determine the compressive modulus. Magnetization analyses were carried out on the fabricated nanocomposite scaffolds using a magnetometer. Magnetization curves as a function of the applied magnetic field were recorded at 37°C. 3D PCL/Fe_3O_4 scaffolds were prepared for cell seeding following a reported protocol.[8] The additive manufactured scaffolds were seeded with bone marrow-derived human mesenchymal stem cells (hMSCs) using 1×10^4 cells per sample. A time-dependent magnetic stimulation of the cell-laden scaffolds were carried out at 1 day after cell seeding. In particular, a sinusoidal magnetic field was discontinuously applied for 6 h per day (20 intervals at 18 min each), taking into account an optimized procedure.[8] Further cell-laden scaffolds were used as controls since they underwent the same conditions without magnetic stimulation. Cell viability and proliferation were analyzed at different time points using the Alamar Blue assay (AbD Serotec Ltd, UK) and the results were reported as a percentage of Alamar Blue reduction. The alkaline phosphatase (ALP) activity was measured with an enzymatic assay (SensoLyte pNPP alkaline phosphatase assay kit – AnaSpec Inc., Fremont, CA, USA). The normalized ALP activity (ALP/DNA) was evaluated by dividing the ALP activity over the DNA content using the Quant-iT PicoGreen assay kit (Molecular Probes Inc., Eugene, OR, USA). The experiments were done at least three times in triplicate. The experimental data were analyzed by ANOVA followed by Bonferroni post hoc test. A value of $p < 0.05$ was considered statistically significant.

6.3 Results

Design strategies and magnetic features play a crucial role in the development of structures for enhanced bone tissue regeneration. [5–8] Currently, the advances in methodologies and design strategies [12–14] have favored the development of high performance devices in different fields. In particular, several efforts have been made to engineer devices for biomedical applications, with a special focus on materials [11,15–17] and experimental [6–8,11–16] studies. The functional behavior of the nanocomposite scaffolds is clearly influenced by the pore spatial distribution, geometrical and architectural features. [5,6] For this reason, design problem, additive manufacturing, experimental and theoretical analyses were the main steps of the current study. The compressive stress-strain curves obtained from experimental tests were consistent with those reported in the literature. [5,6] Compressive modulus and maximum stress are reported in Table 1. Results from magnetic

analyses evidenced a saturation magnetization value of about 4.0 emu g−1. On the other hand, the biological performances of the 3D additive manufactured PCL/Fe_3O_4 scaffolds were also assessed. Figure 1 reports the results as percentage of Alamar Blue reduction over time. The values of Alamar Blue reduction significantly increased ($p < 0.05$) over the investigated time period, thus indicating cells survival and proliferation.

E [MPa]	σ_{max} [MPa]
135 ± 18	14.7 ± 1.6

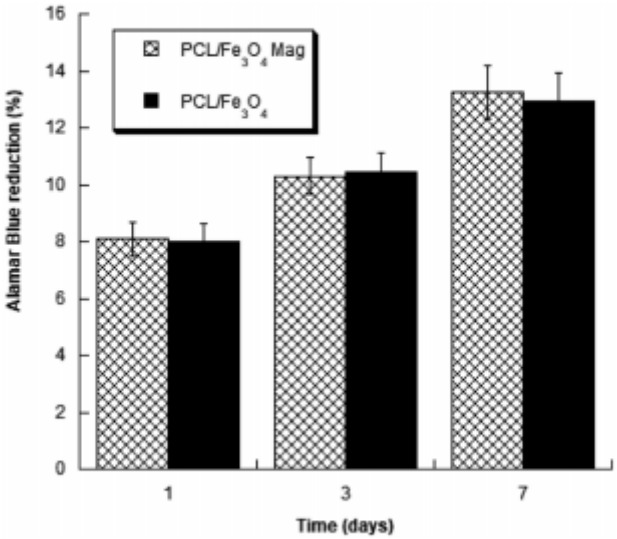

Figure 1. Percentage of Alamar Blue reduction. Data are reported as mean value and error bar represents the standard deviation. The magnetically stimulated PCL/Fe_3O_4 scaffolds are marked as PCL/Fe_3O_4 Mag.

At each time point, no significant differences ($p > 0.05$) were found between the two groups. The ALP activity was measured at different time points for the two groups and normalized to DNA content (ALP/DNA) to assess early osteogenic differentiation. The ALP/DNA significantly increased ($p < 0.05$) over the analyzed time period for the magnetically. stimulated constructs. Such preliminary findings were consistent with those previously obtained for PCL/Fe_3O_4 substrates.[8] Furthermore, in the case of unstimulated PCL/Fe_3O_4 constructs the ALP/DNA values were lower than those found for the magnetically-stimulated PCL/Fe_3O_4 scaffolds at each time point. The observed differences were statistically significant ($p < 0.05$).

6.4 Conclusion

Within the limitations of the current research, the following conclusions were reached: 1) 3D additive manufactured PCL/Fe_3O_4 scaffolds were designed and a further analysis of the role of magnetic features was performed. 2) The combination of the manufactured PCL/Fe_3O_4 scaffolds with the applied time-dependent magnetic field affected cell differentiation.

6.5 References

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Chapter 7 Conclusion

Unseen magnetic forces and their considerable effects still cause scientific disquietude and led to inquire how the cells can be affected by the action of a time-dependent magnetic field. Starting from the data in the literature and due to the confusion of experimental findings is necessary to make a discussion of evidences to clarify the phenomena. Many studies have confirmed the effectiveness of SMFs at the cellular level and it is known that living matter is subjected to the electromagnetic field action mainly by the presence of electric and magnetic properties resultant from mobile ions, spin momentum, paramagnetic elements and the diamagnetic anisotropy.[1] The issue is understand why experimental results present so many controversial reports. At first, there be problems in biological systems to reproduce the same initial conditions tested [2] With respect to the field strength and the exposure time, the results do not seem to follow a proportional relationship and some interaction mechanisms are supposed to work only in very specific SMF intensities, increasing the probability of obtaining different results. The presence or absence of susceptible magnetic elements, such as ferro-paramagnetic components and anisotropic diamagnetic structures, for example, could explain different effects. [3] Many conditions act together to interfere in the cell metabolism and the modification of one simple parameter can bring about a completely different outcome. [4,5] Since the cell and the content of the intracellular environment compose a complex system, the variability of effects would depend on which state the system is at the moment of the magnetic exposure based on the type of cell, the characteristics of the surrounding medium, the presence of paramagnetic elements, the existence of susceptible biomolecules and etc. Some articles mention this phenomenon as a “windows” effect: [6,7] ideal conditions that includes ideal field gradient [8], ideal field intensity [9] ideal exposure duration and ideal organisms exposed. [10] Small variations in the exposure parameters can determine the different endpoint of the experiments, so is care must be taken in relation to the exposure conditions in order to avoid totally different results. Accordingly, the aim of the current research was to analyse novel approaches in tissue engineering, involving the use of magnetic nanoparticles (MNPs) to trigger tissue growth through magnetic fields [11, 12]. Iron oxide nanoparticles have been incorporated into different polymeric matrices (flats, hydrogels, 3D scaffolds) as an advanced method for functionalising biomaterials to guide different kind of tissue regeneration (hard tissue, soft tissue, oral tissue) [13, 14]. Moreover, the superparamagnetic feature of these magnetic nanocomposites has allowed for the induction of a temperature increase (magnetic hyperthermia) through the application of a time-dependent magnetic field [15, 16]. Furthermore, in vitro, the seeding of magnetic labelled cells into a magnetic scaffold has been shown to be efficient. [17]. The fundamental feature of the research was the use of the same parameters of time-dependent magnetic exposure. Regardless of the kind of biomaterial used and the cell lines seeded the magnetic exposure was been always an external sinusoidal discontinuously application for 6 h per day (20 intervals at 18 min each). [18] So, we had the possibility to evaluate the effect of the same magnetic exposure on different multimaterial scaffolds. Magnetic nanocomposite materials offer the unique mechanism to ‘functionalise’ a scaffold by switching an external magnetic field on and off. This feature can be incorporated into the scaffold to provide cue signals that can be activated on demand, thus triggering the sequential biological events that occur during tissue regeneration [19,20].

The introduction of MNPs strongly influenced the mechanical and biological properties of the biomaterials. So, the aim of my PhD project was the analyses of the effects of a magnetic field on the tissue-regeneration process, with a focus on the effects on cell seeding, proliferation and differentiation of human staminal cells. Looking at the overall results obtained, it can be said that the simultaneous presence of magnetic field and magnetic nanoparticles conferred interesting mechano-structural properties to the biomaterials analysed and enhanced the biological properties of human stem cells, stimulating their adhesion, proliferation and the osteogenic differentiation. By exploiting external magnetic fields, this represent a new research area in bone tissue engineering in the direction of a spatial and temporal control of the ongoing tissue regeneration and repair. The optimization of magnetic features and the future research activity will represent the starting point for designing a successful biomimetic structure with “ad hoc” traits for the tissue regeneration.

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