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ELECTROSPUN MEMBRANES LOADING OSTEOGENIC BIOACTIVE MOLECULES FOR GUIDE BONE REGENERATION IN DENTAL IMPLANTOLOGY

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Abstract

The resorption and remodeling of the alveolar ridge after tooth removal due to trauma, periodontal disease, periapical pathology, etc. is a natural healing phenomenon which can negatively impact to the future placement of a dental implant. Because the ridge dimensions are so crucial for decreasing patient morbidity, it is advantageous to preserve the dimension of the post-extraction ridge instead of reconstructing it thereafter, and to maintain its ideal vertical and horizontal dimensions. In order to preserve the ridge volume within the bony envelope existing at the time of extraction, various techniques have been proposed, such as the placement of a bone graft/substitute material into the extraction sockets. However, the quality of the new tissue formed within the socket may vary due to different healing patterns within the alveolar socket with different bone substitute materials. In this context, not only is the amount of the newly formed bone important in these grafted sites, but also the quality of osseous tissues in the socket area is essential, especially when the justification of ridge preservation is to facilitate the placement of a dental implant. Biocompatible materials with high resorption rates, such as poly-lactic acid (PLA), and poly-caprolactone (PCL) allow the formation of bone with no residual graft particles at the time of implant placement, but they are able to limit but not eliminate the post-extraction alveolar ridge resorption to a certain extent.

The present study has been designed to synthesize and evaluate the performance of a new flexible, moldable, electrospun cotton wool-like nanocomposite. This nanocomposite incorporates resveratrol (RSV) into a biodegradable synthetic poly-caprolactone (PCL) or poly-lactic acid (PLA) polymer, and it is prepared through an electrospinning process, which gives it the typical cotton wool-like appearance. This characteristic of the material allows easy proportioning, handling and adaption to a bone defect. In addition, the delivery of RSV, embedded in the polymer, can give new osteoinductive properties to the material. Indeed,

RSV has been shown to have physiological properties that could be useful in regenerative medicine [1]. RSV is a naturally polyphenolic compound present in red wine and in numerous plants and it has been shown to direct mesenchimal stem cells (MSCs) differentiation towards the osteoblast lineage [2] and to stimulate the proliferation and activity of pre-osteoblasts [3]. Moreover, RSV inhibits RANKL-induced osteoclast differentiation and induces apoptosis of differentiated osteoclasts [4]. Although RSV has potential therapeutic application, it is rapidly metabolized and excreted from the body as sulfated and monoglucuronide derivatives, therefore, controlled release of RSV directly at the target site would be more efficient.

Since site specific drug delivery is the best suitable option for bioactive compounds characterized by poor availability, we have developed non-woven RSV loaded and biodegradable nanofiber composite with inbuilt property of high surface area to volume ratio. PCL and PLA have been used specifically as the polymer since they possess remarkable properties like promoting the deposition of extracellular matrix supporting tissue regeneration. Moreover, both polymers can be electrospun efficiently also in the presence of high percentage of RSV, as they show very low viscosity at very high polymer concentration.

Electrospinning conditions were setup to produce a final material composed of individual fibers without any bead formation. The scanning electron microscope (SEM) analysis has suggested that RSV is well dispersed into the materials, resulting in electrospun nanofibers with average diameter around 0.3-0.9 micron.

Normally, drugs encapsulated in nanofibers synthesized through single step electrospinning tend to give initial burst release which may cause reduction in drug therapeutic efficiency.

The *in vitro* drug release profile of the RSV- loaded PCL or PLA nanofibers was studied to test their potential application as Drug Delivery System. The release studies were carried out for a time period of 30 days and the cumulative release behaviors of the drug from the composite nanofibers were analyzed by HPLC. The release pattern for

electrospun nanofibers were studied at two stages: an initial burst release (stage I), followed by decelerate and constant release (stage II). RSV displayed a small initial release of 8,1 % for PCL-RSV and 13,5 % for PLA-RSV within the first 24 hours and thereafter showed a sustained release profile (32 % and 44 % at 30 days).

The *in vitro* osteoinductive efficiency of RSV-released nanofiber on *Dental Puls Stem Cells* (DPSCs) was evaluated analyzing specific differentiation markers such as *runt-related transcription factor 2 (RUNX2), osterix (OSX), osteocalcin (OCN), osteonectin (ONN), osteopontin (OPN) and bone sialoprotein (BSP).* Furthermore, the ability of RSV to inhibit osteoclastogenesis was confirmed by the reduction of RANKL-induced osteoclast differentiation.

In conclusion, our results provide evidence that resveratrol dispersed into electrospun fibers generates bioactive materials able to promote the osteogenic differentiation of mesenchymal stem cells and to inhibit osteoclastogenesis, so they can be useful to improve GBR surgical procedure.

Riassunto

Negli ultimi anni la ricerca sui biomateriali è stata indirizzata verso lo sviluppo e l'ottimizzazione di nuovi *scaffold* per la rigenerazione ossea guidata con particolare attenzione alla progettazione di materiali in grado di indurre e/o mantenere il differenziamento cellulare.

Il design di superfici sempre più simili a quelle del microambiente fisiologico del tessuto osseo (osteomimetiche), mira ad aumentare l'osteoconduttività sostenendo le fasi precoci della cascata di eventi che portano alla rigenerazione tissutale.

Il principio di una rigenerazione ossea guidata prevede il posizionamento di una barriera biologica (membrana) nella cavità alveolare al fine di impedire la sua colonizzazione da parte di cellule non osteogenetiche, come i fibroblasti. In questo modo si viene a creare un ambiente favorevole per la formazione di un fronte neoangiogenico per il differenziamento di cellule perivascolari in osteoblasti [5].

Il processo che porta alla costituzione di un osso rigenerato può essere suddiviso in tre fasi distinte: 1) osteoconduzione, in cui avviene il reclutamento e la migrazione di cellule osteogeniche nel sito anatomico; 2) osteoinduzione, in cui si ha la proliferazione delle cellule progenitrici degli osteoblasti e il successivo differenziamento in elementi maturi osteocitari con deposizione di una matrice extracellulare mineralizzata; 3) il rimodellamento osseo, con la formazione di un osso lamellare maturo in grado di supportare le sollecitazioni meccaniche dell'apparato protesico attraverso una riorganizzazione del tessuto favorita da cicli di apposizione e riassorbimento osseo [6].

Recenti studi hanno dimostrato come una molecola di origine naturale, il resveratrolo, appartenente alla famiglia dei composti polifenolici, rivesta un ruolo determinante nell'induzione del processo di differenziamento delle cellule mesenchimali staminali in cellule osteogeniche, oltre ad avere un ruolo rilevante nei processi di regolazione durante

l’infiammazione. Tuttavia, l’impiego del resveratrolo nel campo della rigenerazione ossea è limitato, non solo dall’eterogeneità delle preparazioni ma anche dall’uso della breve emivita di tale molecola che spesso non consente di ottenere l’effetto osteogenico desiderato [7].

Pertanto, obiettivo del mio lavoro di tesi è stata la realizzazione di membrane biodegradabili a base di poliesteri altamente biocompatibili come il policaprolattone (PCL) e l’acido polilattico (PLA), funzionalizzate con tale fattore osteogenico, al fine di realizzare nuovi “scaffold” in grado di rilasciare molecole bioattive per il differenziamento delle cellule staminali della polpa dentale (*Dental Pulp Stem Cells*, DPSCs) in osteoblasti (Figure 1).

Il PCL è un poliestere alifatico semicristallino che si ottiene per apertura dell'anello del caprolattone e successiva polimerizzazione. Viene già ampiamente utilizzato in medicina per la sua elevata biocompatibilità e la capacità di degradarsi in ambiente fisiologico mediante un lento processo di idrolisi. Inoltre, presenta un'elevata flessibilità per cui può essere ottenuto sotto forma di fibre, film o nanoparticelle [8]. Tale polimero biodegradabile è stato oggetto di numerosi studi per potenziali applicazioni in odontoiatria [9].

Il PLA si è affermato tra i polimeri biodegradabili maggiormente utilizzati per la produzione di impianti e dispositivi riassorbibili. Appartiene anch’esso alla famiglia dei poliesteri alifatici e deriva dall'acido lattico (monomero), ottenuto dalla fermentazione batterica di specifici carboidrati [10].

Le membrane elettrofilate di PCL e PLA sono state preparate utilizzando un processo di elettrofilatura a temperatura ambiente/voltaggio costante e caratterizzate da un punto di vista morfologico utilizzando la microscopia elettronica a scansione (SEM). La cinetica di rilascio del resveratrolo, ottenuta a 37°C in un mezzo minerale con composizione simile a quello salivare, è stata misurata utilizzando la cromatografia liquida ad alta pressione (HPLC). L’effetto della differente cinetica di rilascio del resveratrolo sul differenziamento delle cellule mesenchimali in senso osteoblastico è stato valutato mediante PCR quantitativa utilizzando

markers del differenziamento precoci e tardivi. Come markers sono stati utilizzati il fattore trascrizionale 2 runt-related (RUNX-2), il fattore trascrizionale specifico degli osteoblasti (OSX), l'osteocalcina (OC), l'osteonectina (ONN), osteopontina (OPN) e la sialoproteine Ossea (BSP).

Le micrografie ottenute hanno mostrato membrane di nanofibre di PCL e PLA come una rete di fibre sottili, uniforme, a direzione randomica senza difetti. Dall'analisi al microscopio elettronico il resveratrolo è risultato ben disperso tra le fibre.

La cinetica di rilascio del resveratrolo dalla membrana analizzata mediante HPLC, ha mostrato un rilascio lento e controllato fino a 30 giorni. Dopo 30 giorni di coltura sulle membrane contenente resveratrolo, i risultati ottenuti dimostrano che l'espressione dei markers è significativamente più elevata rispetto al controllo.

Nell'insieme questi dati dimostrano che la funzionalizzazione delle membrane imprime un'attività osteoinduttiva ai supporti sintetizzati, mostrando un'attività più accentuata per la membrana di PCL. In particolare, il resveratrolo rilasciato induce un pattern di differenziamento degli stipiti cellulari presi in esame, concentrazione e tempo dipendente, favorendo una chemiotassi selettiva di cellule progenitrici osteoblastiche nel sito anatomico di inserimento.

Outline of the thesis

The Guide Bone Regeneration (GBR) procedure encourages bone regeneration through cellular exclusion and avoids the invasion of epithelial and connective tissues that grow at the defective site instead of bone tissue. The barrier membrane should satisfy various properties, such as biocompatibility, non-immunogenicity, non-toxicity and a degradation rate that is long enough to permit mechanical support during bone formation. Other characteristics such as tissue integration, nutrient transfer, space maintenance and manageability are also of interest [11].

Nanofibrous membranes developed by electrospinning technology provide attractive conditions for the anchorage, migration, and differentiation of cells. Together with the ease of set up and cost-effectiveness, the possibility to produce nanofibers with a wide range of compositions and morphologies is the merit of this technology. Moreover, by utilizing bioactive natural molecules derived from plants in concert with the nanofibrous matrices, it is possible to provide artificial materials with improved cellular responses and therapeutic efficacy. While there are some challenges in achieving controllable delivery of bioactive molecules and complex-shaped three-dimensional scaffolds for tissue engineering, the electrospun nanofibrous matrices can still have a beneficial impact in the area of regeneration.

Several studies [12] [13] have highlighted the biological effects of resveratrol, showing that this compound could affect mesenchymal differentiation into osteoblasts, inducing tissue regeneration. Indeed, the limit of resveratrol is its rapid metabolism which leads to a poor plasma concentration [14], lower than the concentrations demonstrating *in vitro* effects [2].

The aim of this research project is the synthesis of innovative membranes able to stimulate bone regeneration and also preserve damaged area from possible infections. In particular, the regeneration of the bone tissue induced by resveratrol release from membranes might guarantee the longevity of the treatment.

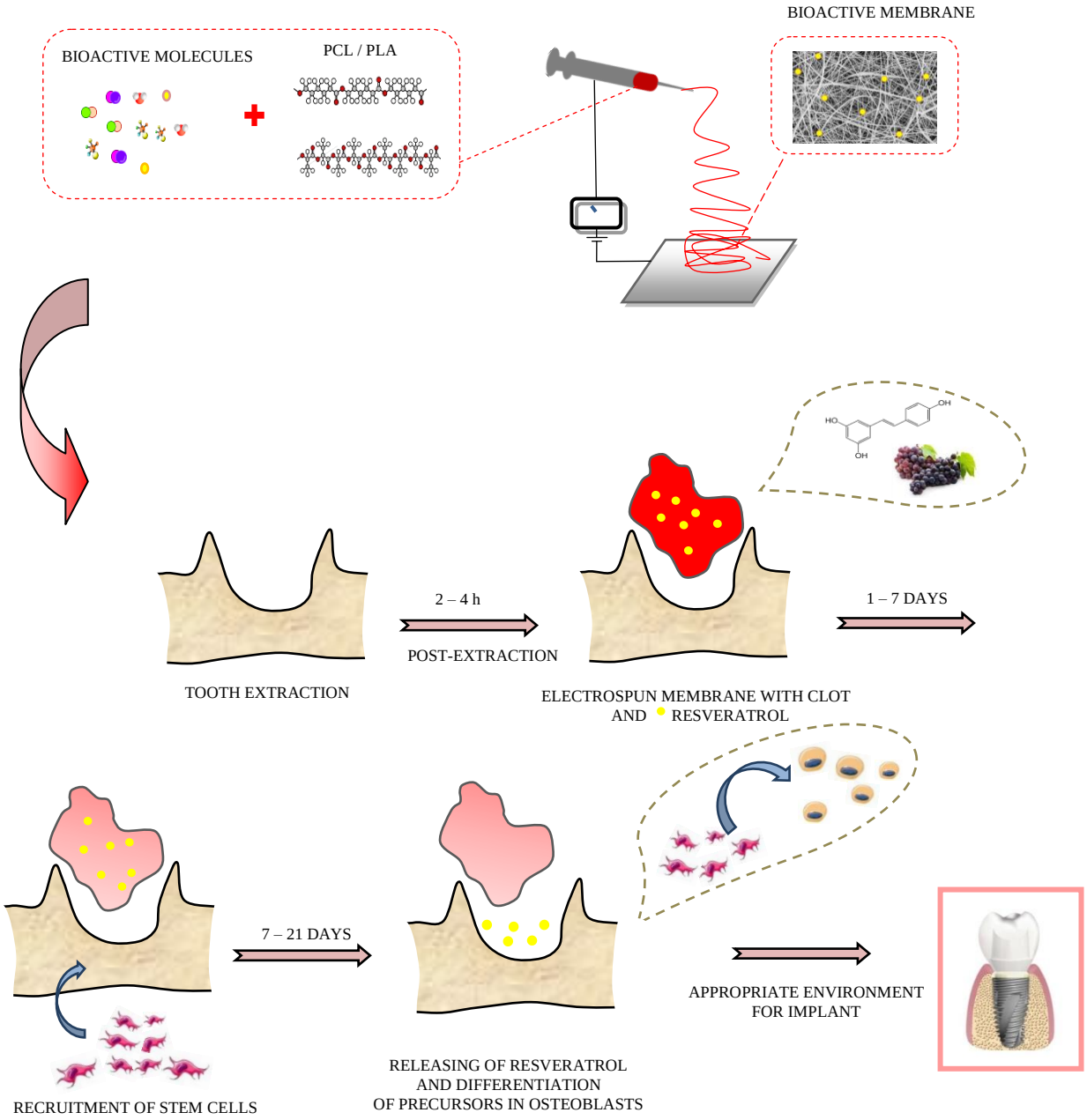

Figure 1. Schematic representation of electrospinning procedure and application of bioactive membrane in the alveolar socket

Introduction

1. Osseointegration in dental implants

Implant bone tooth restorations have become a standard of care in modern dentistry. The presence of sufficient bone volume is an important prerequisite for dental implant placement, for its installation and its successful long-term prognosis. The incidence of failure has been estimated at 10%. However, if more stringent criteria are applied it is likely to be higher. Failure can easily be avoided with proper treatment planning, proper site development, use of surgical guides and a good understanding of the restorative aspects of implant dentistry by the surgeon [15].

1.1 Bone anatomy

Bone or osseous tissue is a dynamic connective tissue able to remodel and rebuild during the lifetime of an individual. It forms the skeleton of an adult human and is composed of different shape and size bones which make up about 15% of our body weight. It is an active tissue responsible for support, protection, locomotion and load bearing. In addition, bone is also involved in hematopoiesis, mineral homeostasis and other functions [16].

Bone is a heterogeneous composite material consisting of organic component, inorganic mineral component and water. Organic component, about 25% of the weight of bone, includes type I collagen (~90%) and other non-collagenous proteins such as sialoproteins and osteopontin [17]. The non-collagenous proteins and proteoglycans cover a small total weight of organic component and they have an important role in osteoblast differentiation and tissue mineralization. The inorganic mineral compartment of bone contributes to ~65% of the bone by weight, primarily in the form of calcium hydroxyapatite (HA) - $Ca_{10}(PO_4)_6(OH)_2$.

Based on its shape it can be classified in long, short, flat, and irregular bone while on its composition, in compact (cortical) or spongy bone (trabecular). Long bones include the clavicles, humeri, radii, ulnae, metacarpals, femurs, tibiae, fibulae, metatarsals, and phalanges while short bones the carpal and tarsal bones, patellae, and sesamoid bones. Flat bones consist

of skull, mandible, scapulae, sternum, and ribs while irregular bones include the vertebrae, sacrum, coccyx, and hyoid bone [18].

Compact bone and spongy bone represent 80% and 20% of the total bone mass, respectively. Compact bone, is formed by cylindrical construction called Haversian or osteons systems and is located in the diaphyseal regions of long bones. The osteons run parallel to long bones and each of them contains lamellae that encircle Harversian canal. Nerves and vessels go through the centric osteons canals whereas nutrients and waste products diffusion is limited. Spongy bone, makes up the inner layer of the bone and is located inside cortical bone in the proximal and distal epiphysis region of long bones and vertebrae [19].

The process that permits to maintain strength and mineral homeostasis in bone is defined as bone remodeling (Figure 2): it begins before birth and continues until death. It increases in perimenopausal and early postmenopausal in women and then continues gradually with aging, but at a faster rate than in premenopausal women. For men, it increases mildly in aging [18]. Therefore, bone results constantly removed (bone resorption) and replaced by new bone (bone formation) and this balance has maintained by two different types of cells: osteoblasts and osteoclasts. Osteoblasts are bone-forming cells, derived from pluripotent mesenchymal stem cells (MSCs) in the bone marrow. MSCs differentiate into osteoblasts, adipocytes or chondrocytes due to activation of specific transcription factors. They form osteoid by depositing extracellular matrix (collagen), then the osteoid becomes mineralised by calcium withdrawn from blood. Some of the osteoblasts differentiate by entrapment into osteocytes [20]. By contrast, osteoclasts are bone cells representative in bone resorption. They derived from hematopoietic progenitors (i.e. monocyte/macrophage) in the bone marrow. Receptor activator of nuclear kappa B ligand (RANKL) produced by osteoblasts bind to RANK receptors located on the surface of hematopoietic cells and promotes their differentiation into osteoclasts [20]. Activated osteoclasts attach to the bone surface and release proteolytic

enzymes that digest connective tissue proteins and solubilize bone mineral. Osteoclastogenesis provides clinical markers of bone resorption; so, in order to compensate bone resorption, osteoblasts also produce osteoprotegerin (OPG) that inhibits osteoclastogenesis by binding to RANKL and blocking interaction with the RANK receptor. Osteoblasts fill the cavity produced by osteoclast-mediated resorption by synthesizing and mineralizing new bone.

Therefore, bone integrity requires a balance between bone-forming osteoblast activity and bone-resorbing osteoclast activity. Anomalies in which bone resorption exceeds formation result in bone loss [21].

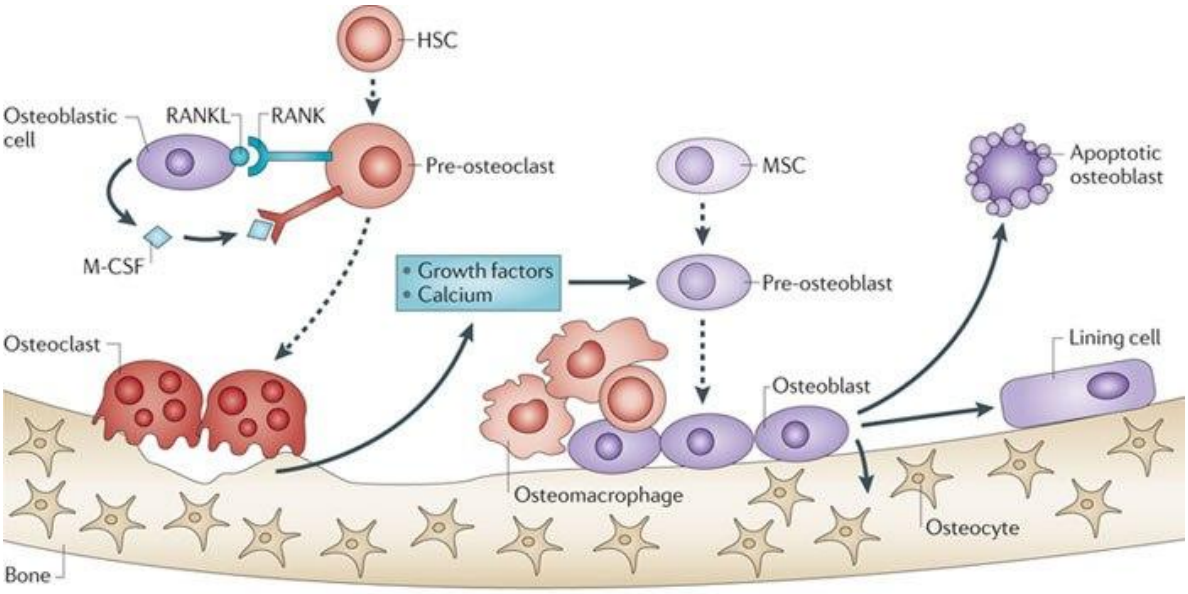

Figure 2. The bone is a dynamic hard tissue that undergoes a continuous remodelling process to maintain skeletal strength and integrity, with 10% of the skeleton being replaced annually. In a finely balanced, coupled and sequential process (indicated by the dashed arrows), haematopoietic stem cell (HSC)-derived osteoclasts resorb bone (releasing growth factors and calcium) and mesenchymal stem cell (MSC)-derived osteoblasts replace the voids with new bone, a process that is dependent on osteoblast commitment, proliferation and differentiation coupled with osteoblast production of type I collagen and its subsequent mineralization to form the calcified matrix of bone. Osteocytes, which are terminally differentiated osteoblasts that are embedded in bone, sense mechanical strain, signal to osteoclasts and osteoblasts, and participate in the remodelling process. Bone lining cells are osteoblastic in origin and have been proposed to form both a canopy over remodelling sites and a layer over bone surfaces, as well as a conduit to communicate with osteocytes. The endosteum and periosteum (the lining on the inner and outer bone surfaces) contain a population of tissue macrophages, termed osteomacs, which are likely to have important roles in bone remodelling. M-CSF, macrophage colony stimulating factor; RANK, receptor activator of NF-κB; RANKL, RANK ligand

Source: Katherine N. Weilbaecher et al., Nature Review Cancer, 2011

1.1.1 Anatomy of maxilla and mandible

Teeth are strongly anchored in the alveolar structure of the jaw bone and they are restricted in the alveolar socket. The joint between the teeth and the alveolar bone is called “gomphosis”. Bone teeth are more elastic in the younger and are more susceptible to pressure so the extraction in these patients is easier. Meanwhile, in the elderly patients, the bone become less vascular and cellular, more compact and do not yield to pressure easily.

The only mobile bone and the heaviest of the craniofacial skeleton is the mandible, also called jaw bone. It consists of a curved, horizontal portion, the body, and two perpendicular portions, the rami, which unite with the ends of the body nearly at right angles [22].

The maxilla, also known as the upper jawbone, is formed from the fusion of two irregularly-shaped bones along the median palatine suture, located at the midline of the roof of the mouth maxillary bones. It is involved in the formation of the orbit, nose and palate, holds the upper teeth and plays an important role for mastication and communication [23].

Mish *et al.* described four bone density groups (D1 to D4) in all regions of the jaws. Dense D1 bone type has a homogeneous cortical structure and it exhibits greater strength than other type, so numerous advantages for implant dentistry. It is often found in anterior mandibles and has fewer blood vessels than the other three types, so the capacity of regeneration is impaired because of the poor blood circulation. D2 is a combination of dense-to-porous cortical bone on the crest and coarse trabecular bone on the inside. This bone type occurs most frequently in the anterior mandible, followed by the posterior mandible. D2 bone provides excellent implant interface healing, and osseointegration is very predictable. D3 is composed of thinner porous cortical bone on the crest and fine trabecular bone within the ridge. It is found most often in the anterior maxilla and posterior regions of the mouth in either arch. The D3 bone is not only 50% weaker than D2 bone, the bone-implant contact is also less favorable in D3 bone. D4 bone, is the opposite condition of D1 and it is located in the posterior region of the maxilla and rarely observed in mandible. Low density and no cortical crestal bone characterized this area [24] (Table 1).

Recent studies have shown that implant therapy in the maxilla has a higher clinical failure than in the mandible. Indeed, in the posterior maxilla it is possible to find thin cortex and low density trabeculae with lower trabecular volume, and a reduction in the thickness and number of trabeculae [25]. On the other hand, results associated with higher survival rate for dental

implants are identified in the anterior region of the mandible, characterized by better volume and density of the bone [26].

TYPE OF BONE	DESCRIPTION
D1 	Homogeneous and compact bone
D2 	A thick layer of compact bone surrounding a core of dense trabecular bone
D3 	A thin layer of cortical bone surrounding dense trabecular bone
D4 	A thin layer of cortical bone surrounding a core of low density trabecular bone

Table 1. Classification of bone density

1.2 Dental implants

A dental implant is a surgical fixture that is placed into the tooth and is able to fuse with bone to hold a replacement tooth or bridge. It provide completely edentulous and partial edentulous patients the function and esthetics they had with natural dentition. Thus, dental implants may be an option for people who have lost a tooth or teeth due to periodontal disease, an injury, or some other reasons.

The primary objectives of implant therapy are two-fold: first, to achieve successful treatment outcomes from a functional, esthetic and phonetic point of view with high predictability and good long-term stability; and, second, to have a low risk of complications during healing and during the follow-up period. The secondary objectives of implant therapy include the fewest possible number of surgical interventions, low pain and morbidity during healing, short healing periods, short overall treatment time and acceptable good-effectiveness [27].

A root-form implant is the most frequently used type of dental implant today and consists of three main components: fixture, abutment, and prosthesis. The fixture is a cylinder-shaped metal post that is surgically embedded into the osseous portion of the jaw, simulating the shape of the root of a tooth. After surgical insertion, the top of the fixture will be flush with the surface of the alveolar bone. The abutment is attached to the fixture using an abutment screw, which raises it from the bone surface to above the mucosal surface [28].

In addition, there are two different types of dental implants:

Endosteal (in the bone) which includes screws, cylinders or blades surgically placed into the jawbone. Each implant holds one or more prosthetic teeth. This type of implant is generally used as an alternative for patients with bridges or removable dentures.

Subperiosteal (on the bone) which is placed on top of the jaw with the metal framework's posts protruding through the gum to hold the prosthesis. These types of implants are used for patients who are unable to wear conventional dentures and who have minimal bone height [28].

The biomaterials used for manufacturing dental implants include metals, ceramics, carbons, polymers, and combinations of these. Polymers are softer and more flexible than the other classes of biomaterials. They also present with low mechanical strength, which make them prone to mechanical fractures during function under high loading forces. Polymeric materials were reported to have very little application in implant dentistry and were only used to fabricate shock-absorbing components placed between the implant and the suprastructure [29]. Ti, including alloy Ti-6Al-4V (Ti-6 aluminum-4 vanadium), is the first modern material used for dental implants, and it is still one of the most used in contemporary dental implants. Other metals have been used for osseointegration, including zirconium, gold and Ti-aluminum-vanadium alloys. These alloys may strengthen the implant but have been shown to have relatively poor bone-to-implant contact [30].

Bioceramics such as hydroxyapatite are also used because although their low strength, excellent biocompatibility, and capacity to integrate with hard tissue and living bone. Besides their brittle nature, hydroxyapatite, tricalcium phosphate, and aluminum oxide ceramics are currently used as plasma-sprayed coatings onto a metallic core [31].

For dental implants to be successful, tooth must have enough bone to support them. Tooth loss often leads to more loss of bone over time. The tooth loss may be caused by:

- Periodontal disease
- Dental caries and infection
- Injury or trauma
- A defect in development

Statistics provided by the American Association of Oral and Maxillofacial Surgeons show that 69% of adults ages 35 to 44 have lost at least one permanent tooth to an accident, gum disease, a failed root canal or tooth decay. Furthermore, by age 74, 26% of adults have lost all of their permanent teeth. Therefore, in patients with orofacial pain, dental implants may resolve painful symptoms as well as improve facial esthetics and appearance. Edentulous patients may gain a feeling of higher selfesteem and well-being. In patients with craniomaxillafacial defects, implants can be used to replace ears, noses, eyes, and other maxillofacial defects. Moreover, congenital, traumatic, and developmental oral defects can be treated with implants [27].

1.2.1 The concept of osseointegration

Osseointegrated implants have been used to replace missing teeth and as anchorage for orthodontic tooth movement with direct bone contact. The concept of osseointegration was originally introduced by Brånemark *et al*. in 1969. He introduced a new system of dental implants later regarded as a clinical achievement. He observed that a piece of titanium embedded in rabbit bone became anchored and difficult to remove with direct bone contact.

No inflammation was detected in the peri-implant bone after 1 year; meanwhile, soft tissue had formed an attachment to the metal and bone to the titanium. This discovery produced a breakthrough in dentistry field introducing the term of osseointegration to describe this phenomenon [32]. Albrektsson *et al.* [33] suggested that this was "a direct functional and structural connection between living bone and the surface of a load carrying implant." Another clinical definition provided by Zarb and Albrektsson [34] proposed that osseointegration was "a process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading."

Nowadays osseointegration can be defined as a direct and functional anchorage between living bone and the surface of a load-carrying implant. It has considered a prerequisite for implant loading and long-term clinical success of endosseous dental implants. The process is quite complex and dependents on the interrelationship of the various components such as the implant material, macroscopic and microscopic nature of the implant, the surgical technique, the undisturbed healing phase and loading conditions [35].

It involves two different phases: firstly, an initial interlinking between alveolar bone and the implant body that creates a direct apposition of bone to the implant surface without any interposing collagen or fibroblastic matrix. Subsquently, a biological fixation through continuous bone apposition and remodeling toward the implant. So, the bone has all characteristics of living bone, such as osteocytes or blood vessels, close to the implant surface. Implant stability, which occurs after implant integration, is an indirect measure of successful osseointegration and it can occur at different stages, primary and secondary stability.

Primary stability is associated with the mechanical involvement of an implant with the surrounding bone. It has defined as the biometric stability immediately after implant insertion and results a critical factor that determines the long-term success of dental implants.

Subsequently, bone regeneration and remodeling phenomena determine the secondary (biological) stability [36].

1.3 General factors contributing to implant failures

Dental implants have become important therapeutic tools in the last decades and their success rates are 85–95%. However, implant failure can occur despite adequate surgical conditions [37]. The successful outcome of dental implants depends on a series of parameters such as implant geometry, biocompatability of the implant material, surgery techniques, quality and quantity of local bone oral but also from patient-related elements such as general health, systemic disease, smoking, unresolved caries or infections. So, inappropriate conditions may contribute to implant failure. According to Esposito *et al.* [38], implant failure can be divided into biological failures which include "early failures" and "late failures". Early failure refers to all conditions which interfering with the initial bone healing process, such as poor bone quality and density, patient medical conditions, sign of infections, lack of implant stability. Meanwhile, peri-implantitis seem to be the most important factors associated with late failure.

1.3.1 Bone quality

The knowledge of quality bone at the implant site results an important factor in determining the success of dental implants; it is believed to be one of the most important aetiological factors for early implant failures. The first classification for bone quality has been attributed to Lekholm U, Zarb GA (1985), who explained its classification system based on their radiographic appearance. Type 1 is a bone in which the entire bone is composed of homogenous cortcal bone while type 2 in which there is thick cortical bone with marrow cavity. Type 3 and type 4 bone are characterized by thin cortical bone with dense trabecular bone of good strength and very thin cortical bone with low density trabecular bone of poor strength, respectively [39].

Analysis of the reviewed studies showed that the outcome of implant treatment could be related to bone quality. It was observed by many studies that there is an increased implant failure rate when implants are inserted in bone qualities type III and IV [40] [41] [42] or in bone quality type IV [43] [44] [45]. Few studies reported a higher implant failure rate for implants inserted in bone quality type II (or type III) [46] [47]. Some observed that insertion in bone type I (or 'dense bone') may also result in an increased implant failure rate [48] [45].

The bone quality type I is more commonly found in the anterior mandible, where usually there is more bone available to insert long implants. Thus, this may be related to overheating of the bone when long implants are placed. Research has demonstrated that thermal damage at the drilling site inhibits the regenerative response in bone healing, slowing the process of osseointegration and potentially resulting in implant mobility [49].

1.3.2 Patient medical conditions

Nowadays, the increasing attention in dentistry implantation has been focused on healthy patient-related conditions which could influence implant survivability by interfering with the tissue healing process. Conversely, the role of age in implant failure, results unclear. Some authors suggest how people over 60 have an elevated risk of failure while other report that the age has a minor effect [50]. Certainly, with advanced ages, change which occurs in composition of bone, may take longer to heal in patients older.

Osteoporosis, which is a disorder characterized by a generalized diminution in bone mass and bone density, may therefore represent a risk factor for osseointegration [51]. As the prevalence of osteoporosis rises with age, some researchers investigated whether the age of the patient may have some influence on the implant failure rates. Few studies showed that there is an increase in implant failures with age [52]. On the other hand, most of them have shown that there is no correlation between implant failure and age of the patient [53] [54] [55] [56].

Systemic diseases, such as immunological and malabsorption disorders, lupus, lichen planus, could interpose the outcome of implant restoration. Several studies have also reported that persistent hyperglycemia in diabetic individuals, inhibits osteoblastic activity, alters metabolism of Ca and P, decreases collagen formation during callus formation, induces apoptosis in bone cells and increases osteoclastic activity. In particular, high percentage of early failure of implants in diabetics compared to late failure [57] indicates increased failure rate within first year of loading [58].

The literature suggests that the rate of implant failure is higher in irradiated bone. So, patients expose to chemotherapy and/or radiation therapy for oral or head cancer can have a limited amount of bone, making it very difficult to place dental implants [59].

Furthermore, patients with a history of cardiovascular events such as recent stroke and a cardiovascular surgery, might represent an absolute contraindication to implant therapy [60].

1.3.3 Infection in situ and lack of primary stability

Infection is currently regarded as the most severe and devastating complication contributing to implant failure. There is not a single microorganism associated with colonization of infection, but it would be more appropriate refer to a microbial flora [61]. Indeed, staphylococci, coliforms and Candida are commonly isolated from peri-implant lesions [62].

In particular, Staphylococcus aureus and coagulase-negative staphylococci are frequently involved in infections with metallic biomaterials or medical infections in general. Harris LG *et al*. demonstrated that Staphylococcus aureus was able to adhere properly to titanium surfaces, highlighting relevance of this gram negative bacteria in the colonization of dental implants and subsequent infections [63]. So, in order to reduce microbial contamination in dental implant, several strategies have been postulated during surgery, such as rinsing preoperatively with chlorhexidine; an in-vivo study by Noiri *et al.* has showed that chlorhexidine in suspension form is more effective in inhibiting Porphyromonas gingivalis in

comparison to antibiotics [64]. Recently, the interest for anti-infective biomaterials in implantology has represented a real progress, proved by the number of published papers on this topic. So, several diversified strategies have been developed to create materials with bactericidal activity able to interfere with microbial colonization and to disrupt the structural integrity of single bacterial cells and of pathogens [65] [66]. Furthermore, although the risk of developing an implant-related infection is highest for events originated during surgery, a residual risk still remains for the possibility of late infections from distant colonized anatomic sites. Thus, much has been done in terms of prevention.

Another fundamental prerequisite for implant success is the primary stability at the time of insertion. It results as a critical factor because determines the long-term success of dental implants. Lack of primary stability, defined as a markedly mobility of implant in the bone bed, is one of the major cause of its failure [67]. Movements even of micrometers can induce a stress: a micromotion between 50 and 150 mm may negatively influence osseointegration and bone remodelling by forming fibrous tissues at implant interface [68].

1.3.4 Perimplantitis

Late failures may be subclassified into late early or late delayed depending on failures occur during or after the first year of loading [69]. They are both characterized by changes in loading conditions and peri-implantitis.

Peri-implantitis is an inflammatory condition affecting the tissues around an implant in function, causing loss of supporting bone and in the end the failure of osseointegration [70]. They have been reported in 5-8% of cases within selected implant systems and bacterial infection seems to be the etiology of the disease [71]. This inflammatory condition is based on the clinical signs at implant site, such as hyperplastic soft tissues, suppuration, color changes of the marginal peri-implant tissues and gradual bone loss. These signs are identify both clinically and radiographically measuring clinical parameters including peri-implant loss of

gingival attachment, bleeding on probing, plaque/gingivitis indices, suppuration and mobility [72].

2. Biology of healing and bone regeneration

The normal healing process after tooth removal is a multistep repair that follows a determined spatial and temporal sequence. It starts with an inflammatory phase, characterized by the formation at the site of injury of a blood clot rich of blood products, such as platelets, leukocytes, macrophages, fibrin, soluble growth factors and cytokines. These events begin within the first 12 to 14 hours after the injury, with a peak during the first 24 hours and are completed around 7 days. Firstly, neutrophils, and subsequently macrophages and lymphocytes arrive at the site of injury. Macrophages phagocyte necrotic tissue and contemporary release growth factors and cytokines that initiate the healing process of bone wound [73]. The factors secreted by these cells stimulate the migration of multipotent stem cells from the surrounding tissue and induce their differentiation into the osteogenic or chondrogenic lineages. During following days starts a construction phase in which increases the production of blood vessels from pre-existing vessels. Local vascularization at the site of injury has one of the most important parameters that influences the healing process allowing nutrients and osteoblast precursor cells arrival [74] [75]. In this phase intramembranous and endochondral ossification generates new bone formation: during intramembranous ossification bone is formed directly without first forming cartilage. Migrated mesenchymal stromal cells that reside in the periosteum directly differentiate into osteoblasts and synthesize and deposit bone matrix. This process creates a callus formation, histologically defined as ‘hard callus’. By contrast, endochondral ossification involves the recruitment, proliferation, and differentiation of undifferentiated mesenchymal cells into a transient cartilaginous matrix, which calcifies into mature bone. Contributes from the adjacent to the fracture periosteum and external soft tissues, providing an early callus, described as ‘soft callus’ that stabilizes the fracture fragments and will act as a template for subsequent mineralization [76].

The last phase of healing process called remodeling phase, occurs over the course of months and involves restoration of original structure and strength bone. During this phase, osteoclasts reabsorb and form bone tissue while osteoblasts deposit more osteoid and calcium phosphate, increasing the density of mineralized matrix. So, the density of internal structure increases gradually [77] (Figure 3).

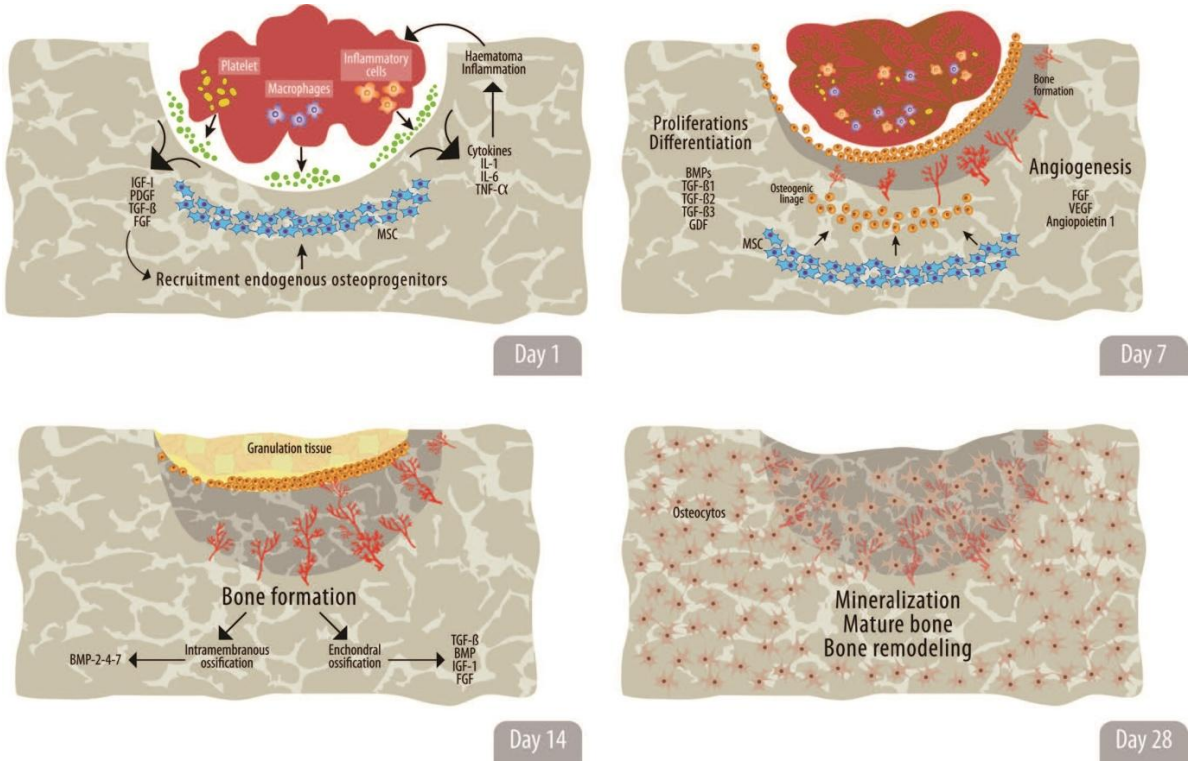

Figure 3. Temporal progression of bone healing. The healing response to bone injury is characterized by overlapping biological processes: immediately after bone injury, hematoma formation and inflammatory response permits the re-lease of pro-inflammatory cytokines and growth factors that initiate the process of wound healing. Between days 1–7, MSCs proliferate and differentiate into the osteogenic or chondrogenic lineages and increase the production of blood vessels from pre-existing vessels. New bone formation occurs through intramembranous or endochondral ossification that is finally mineralized, forming a mature bone that is continuously remodeled through the rest of his life

Source: Orlando Chaparro and Itali Linero, Advanced Techniques in Bone Regeneration, 2016

2.1 Biological actors in bone regeneration

Bone regeneration is characterized by the temporal release of signaling molecules able to regulate and influence cellular responses interfering with differentiation and proliferation.

They directly affect the bone formation, increase the number of bone forming progenitor cells [78] and interfere with normal process of bone regeneration coordinating the healing cascade events [79].

These biological molecules can be classified into three different categories: pro-inflammatory cytokines, growth and differentiation factors and angiogenic factors (Table 2).

Pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-11, IL-18 and tumor necrosis factor-α (TNF-α) belong to first group. They are critical after bone injury because establish an adequate environment for the initial phase. They have a chemotactic effect on other inflammatory cells, enhance ECM synthesis, stimulate angiogenesis, and recruit endogenous fibrogenic cells to injury [79]. In particular, TNF-α is able to recruit mesenchymal stem cells (MSCs) and has a prominent role during endochondral ossification, while IL-1 is mainly produced by osteoblasts and is involved in bone remodeling [80].

The second group consists of growth and differentiation factors which are secreted during the constructive phase of bone regeneration and includes the super-family of transforming growth factor-beta. They cover a large group of regulatory molecules such as bone morfogenetic proteins (BMPs) and different isoforms of transforming growth factor-βs (TGF-β).

BMPs are pleiotropic factors and act in regulating the formation, maintenance and bone repair [81]. The physiological role of these factors results difficult to define because of their functional redundancy. Indeed, their effects depend on the target cells, stage of differentiation, local concentration, as well as interactions with other secreted proteins [82]. However, has been shown that BMP 6, and BMP 9 may act as inductors of osteoblast differentiation of mesenchymal progenitor cells [83] while, BMP 2, BMP 4 and BMP 7 may have a prominent role during bone regeneration process. In details, BMP 2 results active before formation of immature bone structures during both endochondral and intramembranous ossification [84]. BMP 4 is more active from 1-5 day after injury while BMP 7 after 14 days [85].

Transforming growth factor-βs (TGF-β) is a multifunctional protein with five isoforms which regulates differentiation and cell proliferation of MSCs, pre-osteoblasts, osteoblasts and chondrocytes. In addition, it stimulates the extracellular production of proteins such as collagen, proteoglycans, osteopontin, osteonectin and alkaline phosphatase.

According to Tsiridis *et al.*, it may induce the synthesis of BMP by osteoprogenitor cells during endochondral bone formation and inhibit proliferation and differentiation of osteoclasts [76].

Other differential factors include platelet derived growth factors (PDGFs), fibroblast growth factors (FGFs), Insulin-like growth factors (IGFs). PDGF is synthesized by several cells, including platelets, monocytes, macrophages, osteoblasts, and endothelial cells and is the major stimulus for MSCs and osteoblasts [86]. FGFs consist of nine structurally related polypeptides involved in early stages of fracture healing, in angiogenesis and mesenchymal cell mitogenesis [87]. Finally, IGFs consist of two different forms, I and II: IGF-I promotes bone matrix formation while IGF-II is implicated in the late of endochondral bone formation [88].

The last group of molecules includes angiogenic factors, key elements for the vascularization of the wounded area. Vascular endothelial growth factors (VEGFs) and matrix metalloproteins (MMPs) work together in order to degrade cartilage and bone, allowing the invasion of blood vessels [89].

SIGNALING MOLECULES	CLASSIFICATION	FUNCTION
PRO-INFLAMMATORY FACTORS	IL (1-6-11-18), TNF-α	Bone injury, chemotactic effect on other cells, endochondral ossification, angiogenesis, ECM synthesis
GROWTH/DIFFERENTIATION FACTORS	TGF-β, BMPs, PDGF, FGFs, IGFs	Differentiation of MSCs into osteoblasts, regulation of formation and bone repair, bone regeneration process
ANGIOGENIC FACTORS	VEGF, MMPs	Angiogenesis in healing process, mobilization and recruitment of endothelial progenitors cells, matrix degradation

Table 2. Biological actors in bone regeneration

2.2 Current treatment for bone regeneration: bone grafts

In the last decade, an important challenge in oral maxillofacial surgery has been to create novel strategies to improve clinical outcomes in this field. In oral dentistry, different treatments have been developed to correct bone defects, which are the cause of functional disability. Both clinical and preclinical research continues to evaluate advanced regenerative approaches using bone grafts materials, new barrier membranes, cell-growth-stimulating proteins in order to rebuild the missing support or correct alveolar bone [90].

Recently, several tissue engineering approaches have been attempted based on mesenchimal stem cells and biocompatible materials with or without growth factors but also with delivery approaches or gene therapy applications [91].

The potential role of bone grafts to promote healing depends on their osteoconductive and osteoinductive properties, by their origin and composition. Nowadays, they are a therapeutic strategies widely used for the correction of osseous defects. Four graft materials are commonly used for clinical application: autologous grafts, allografts, xenografts, and synthetic grafts or alloplast.

Autologous graft is referred to tissue transferred from one location to another within the same individual. It is a clinically approved therapy and shows the biological characteristics of osteogenesis, osteoconduction, and osteoinduction. Autogenous bone can be harvested from different portions, not only extra oral sites such as the iliac crest (gold standard source) but also intra oral sites such as the mandibular symphysis, maxillary tuberosity [92]. It is considered a rich source of progenitor osteogenic cells but presents some limitation: the volume of bone may be limited so is necessary to harvest portion from secondary surgical site [93]. In addition, other disadvantages are morbidity of the donor site, prolonged surgical time and graft resorption [94]. So, these limitations have led to investigate another kind of graft, allograft bone, which consists on bone harvested from another individual of the same species. It has osteoconductive characteristic, but presents less osteoinductive properties. Bone allografts eliminate the possibility of a secondary donor site, have reduced surgical time, decresed blood loss and host morbidity. In addition, can be used in different forms, such as mineralized, demineralized, frozen or freeze-dried bone [11].

Xenografts are graft materials mainly derived from animals, such bovine or porcine sources. The forms frequently used are natural hydroxyapatite and deorganified bovine bone. Disadvantages of this graft is the risk of a host-immune response, so in order to prevent possible rejections, the proteins can be removed with different procedure [95]. Furthermore, xenografts result integrate into natural bone, but their low resorption rate may compromise the healing of the grafted site and the properties of the regenerated bone.

In order to overcome this limitation, in the last decade alloplastic bone substitute materials have been introduced as a promising alternative. They include synthetic materials such as porous and non-porous Hydroxyapatite (HAp), HAp cement, β-tricalcium phosphate (TCP), polymers, bioactive glasses calcium-based ceramics, calcium-sulphates and bioactive glass. They are wildly used in orthopaedic practice because reduce the problem of limited supply of

autografts bone and the risk of disease transmission derived from allografts and xenografts. The use of alloplast grafts to treat oral disease appears promising but histologically they present some limitations: the grafts remain in situ for long time and they are encapsulated by fibrous tissue rather than resulting in true bone formation. Moreover, for clinical application, these materials need to be improved [96].

3. Electrospun nanofibers for bone regeneration

In last decade, electrospinning has emerged as an extremely promising method for the preparation of tissue engineering scaffolds. The fabrication of nanofibers has attracted the interest of researchers due their unique property required for biomedical applications. In particular, elettrospinning is used to create nanofibers for various dental applications such as tooth regeneration, wound healing and prevention of dental caries, repair and regeneration of dental and oral tissues including dental pulp, dentin, periodontal tissues, oral mucosa and skeletal tissues [97].

In recent years, biodegradable polymers have attracted considerable attention as biomaterials in pharmaceutical, medical, and biomedical engineering applications, including drug delivery systems, artificial implants, and functional materials in tissue engineering. Aliphatic polyesters, in particular, due to their favorable features of biodegradability and biocompatibility, comprise one of the most important classes of synthetic biodegradable polymers. The advantage of these polyesters is their biocompatibility and higher hydrolysability in the human body [98].

3.1 Set up condition of elettrospinning

Electrospinning (also termed electrostatic spinning) has gained substantial attention in the last two decades triggered by the potential applications of electrospun nanofibers in nanoscience and nanotechnology [99]. Particularly, remarkable features such as large specific surface area, high porosity, and spatial interconnectivity of electrospun nanofibers make them well suited for nutrient transport, cell communication, and efficient cellular responses [100, 101]. Furthermore, nanofibrous structures developed by this technique provide attractive extracellular matrix conditions for the anchorage, migration, and differentiation of tissue cells, including those responsible for the regeneration of hard tissues. Together with the ease of set

up and cost-effectiveness, the possibility to produce nanofibers with a wide range of compositions and morphologies is certainly its merit.

The apparatus of elettrospinning consists on a syringe with a metallic needle, a counter electrode (normally a metal plate), a source of electrical field (high voltage supply) and a pump (Figure 4). An electric field (usually of 10-30 kV) is applied to a polymeric solution that comes out from the tip of a metallic needle, which acts as one of the electrodes. The high voltage applied leads to deformation of the solution drop (called Taylor cone) and finally to the ejection of a charged polymer solution jet from the tip of the cone, accelerating towards a counter-electrode. The jet undergoes an instability and an elongation process, becoming very long and thin. In the meantime, the solvent evaporates leading to the formation of continuous solid fibers. Generally, the counter-electrode is placed at the distance of 10-30 cm from the needle tip and fibers are collected on its surface as a nonwoven mat [102].

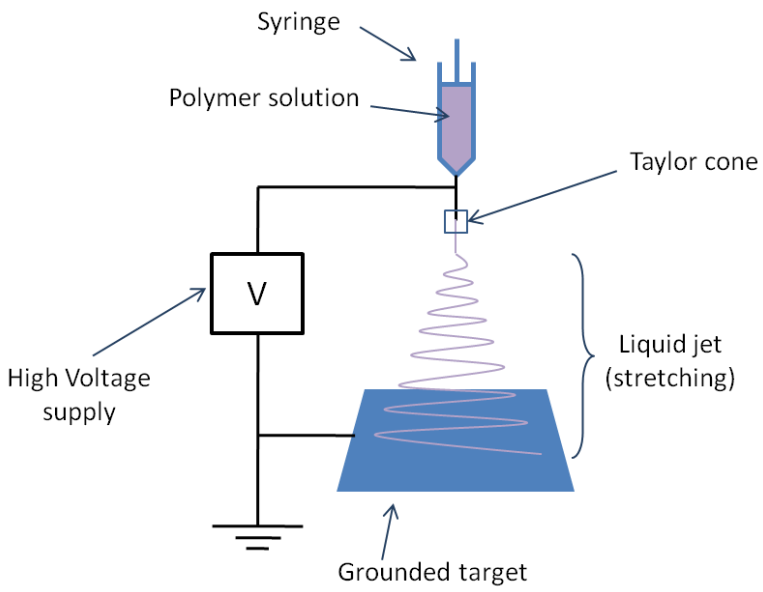

Figure 4. Example of electrospinning apparatus

Source: V. Salles, Nanotecnology and Nanomaterials 2012

3.2 Electrospinning process variables

Different variables could alter the morphology of electrospun materials, due to the complexity of the electrospinning process. These variables can be divided into two categories:

- properties of the polymer solution
- parameters of the electrospinning equipment.

Solution parameters

The choice of polymer and solvent, polymer molecular weight and polymer concentration are considered controllable variables of the polymer solution. The choice of polymer should always be dictated by the desired final product while the solvent chosen must be compatible with the polymer. In addition, solvent choice will have an impact on properties such as solution conductivity, surface tension, and evaporation rate.

The molecular weight of the polymer primarily affects the mechanical properties of the polymer solution, including the viscosity and relaxation time. The concentration of the polymer, combine with the polymer molecular weight, will determine the number of entanglements per polymer chain, which has been implicated in the ability of the solution to be electrospun [103]. So, the concentration of polymer also has an important role in the evaporation of the solvent: more polymer there is in the solution, the less solvent there is to evaporate before solid fibers are formed. Finally, additives can be used in the solution to change its properties without changing the polymer component, improving in this way its ability to be electrospun.

Processing parameters

The volumetric flow rate of solution to the needle tip, the applied voltage, the distance from the needle tip to the collecting target, the configuration and rotational velocity of the collecting target, and finally temperature of the apparatus are all parameters which can affect the elettrospinning process and alter the morphology of the final product.

The flow rate determines the amount of solution available for electrospinning. Maintaining a stable Taylor cone requires a minimum solution flow rate for a given voltage and electrode gap [104]. At low flow rates, the Taylor cone recedes into the needle, and the jet originates from the liquid surface within the needle. In contrast, if the solution flow rate is greater than the electrospinning rate, it causes solution droplets to fall from the needle tip because of lack of time for electrospinning the complete droplet to be electrospun. It has been observed that the diameter of the fibre and the size of the bead both increase with an increased flow rate.

The applied voltage can influence the charge density on the polymer solution surface; generally, either a positive or a negative voltage of more than 6kV is required in order to cause the jet to initiate from the Taylor cone. However, if the applied voltage is higher, a greater amount of charge will cause the jet to accelerate faster, and more solution will be drawn out from the tip of the needle [105].

The distance between the needle tip and the collecting target can also affect the strength of the electric field produced. This distance will also dictate the amount of time that the solvent has to evaporate. Furthermore, the rate of evaporation depends on the polymer and solvent choice and polymer concentration, as well as the ambient conditions, such as temperature and humidity. High temperature and low humidity will promote fast evaporation of solvent and solidification of the polymer jet. If the time of flight and evaporation kinetics of the polymer solution is not sufficient for complete drying, wet fibers could be deposited that will flow and bond with each other. Finally, the arrangement and alignment of the fibers can be altered by changing the geometry of the collecting target; for example, a rapidly rotating target can align the collected fibers [106] [107].

3.3 Electrospun polymers for tissue engineering

One of the most attractive aspects of the electrospinning is its versatility. A large number of polymers have been electrospun, including biopolymers like collagen, silk, and hyaluronic acid, hydrophilic polymers such as poly(ethylene glycol) and poly(2-hydroxyethyl methacrylate), and hydrophobic polymers like poly(lactide-co-glycolide) and poly(caprolactone). So, these wide range of polymers capable of being electrospun are possible approaches to bone tissue engineering and give researchers flexibility in creating fibers with diameters in the nanometer to micron range [97].

Specifically, there are two groups of polymers that are used: synthetic and natural. A variety of natural polymers such as collagen, gelatin, elastin, silk, and synthetic polymers such as poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(e-caprolactone) (PCL) and poly(lactic-co-glycolic) acid (PLGA) have been electrospun as biomimetic to modulate various cellular activities. However, synthetic or natural polymer alone cannot meet all the requirements for tissue engineering. Synthetic polymers have great flexibility in synthesis and modification, but these polymers lack cell affinity because of their low hydrophilicity and lack of surface cell recognition sites. Compared to synthetic polymers, natural polymers provide good biocompatibility but tend to display poor processing ability and mechanical properties [101]. Therefore, it is desirable to fabricate composite fibrous membranes which might possess not only suitable mechanical properties but also bioactive characteristic for cellular adhesion, proliferation, and differentiation [108]. So, to develop biomimetic bone tissue engineering scaffolds for tissue engineering, specific bioactive molecules can be incorporated into the polymer to create their controlled delivery system.

3.3.1 Poly lactic acid (PLA)

Poly(lactic acid) (PLA) is a degradable aliphatic polyester that can be produced synthetically or from renewable resources such as whey or corn.

PLA can be synthesized through two processes; direct polycondensation of lactic acid and ring-opening polymerization of lactide, a ring oligomer of lactic acid [109].

The monomer consists of two stereoisomeric forms: L-lactide and D-lactide. The most common occurring form is the L-lactide; D-lactide is not naturally occurring, it is expensive and not regularly used in research. They give origin to the two homopolymers, poly-L-lactic acid (PLLA) and poly-D-lactic acid (PLDA), respectively.

PLA is naturally hydrophilic due to its polar oxygen linkages. It contains a methyl side group, which confers hydrophobic properties to this polymer (Figure 5) [110].

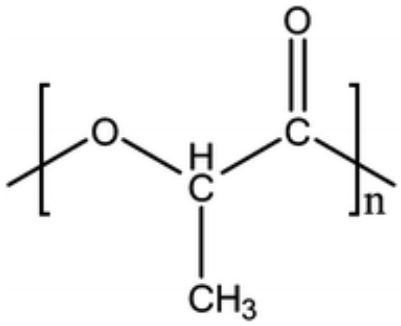

Figure 5. Structure of PLA

The natural hydrophilic characteristic of PLA is responsible for its moderate decomposition in accordance with the surrounding moisture and temperature. The first stage of PLA degradation is usually the reduction of its molecular weight by hydrolysis to < 10 kDa before it becomes biodegradable. The hydrolysis of PLA occurs by random cleavage of the –C–O– ester bond by water molecules. The hydrolysis products, which may contain fragments of lactic acid, oligomers and other water soluble products, can then be consumed by microorganisms to produce carbon dioxide (CO_2), water (H_2O) and solid biomass [111].

3.3.2 Poly(ε-caprolactone) (PCL)

Poly(ε-caprolactone) (PCL) is a synthetic biodegradable aliphatic polyester derived from the ring opening polymerisation of ε-caprolactone (Figure 6).

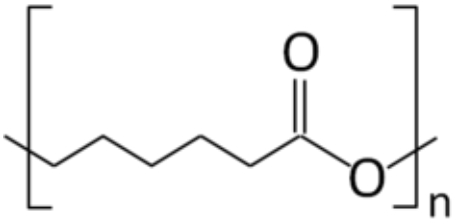

Figure 6. Structure of PCL

The polymer can be synthesized in different ways, including the use of enzymes, metal catalysts and organic methods but ring opening polymerization is the most common methodology due to the cost and quality of the product.

PCL is degraded by hydrolysis of its ester linkages in physiological conditions (such as in the human body) and it is often used for the preparation of long term implantable devices. Indeed, its degradation is even slower than that of polylactide (the degradation time of the bulk polymer is longer than PLA, 30 months compared to 20). Literature reports that PCL undergoes a two-stage degradation process: firstly the non-enzymatic hydrolytic cleavage of ester groups and subsequently, when the polymer is more highly crystalline and has a low molecular weight (less than 3000) is shown to undergo intracellular degradation. This was observed during experiments of PCL fragments uptake in phagosomes of macrophages and giant cells and within fibroblasts [112] and supports the theory that PCL may be completely resorbed and degraded via an intracellular mechanism once the molecular weight was reduced to 3000 or less. So, the mechanism of PCL degradation could be attributed to random hydrolytic chain scission of the ester linkages, which caused a decrease in molecular weight.

In addition, porosity, crystalinity, surface area and molecular weight all play critical roles in the time required for the polymer to degrade.

3.4 Polyglycolic acid (PGA)

Polyglycolic acid (PGA) is a linear aliphatic polyester of the poly(α-hydroxy esters) family and it is produced by ring-opening polymerization of cyclic diesters of glycolide (Figure 7). It is a semicrystalline polymer with a melting point between 185 and 225°C, a glass transition of 36-40°C and a low solublility in organic solvents. PGA is hydrophilic and undergoes bulk degradation with glycolic acid release, which is metabolized by the body. The drawback of glycolic acid is the possible local inflammation in the surrounding tissue, so, it is not so used for tissue engineering [113].

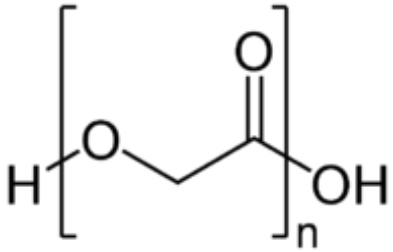

Figure 7. Structure of PGA

3.5 Guide Bone Regeneration (GBR)

Guided bone regeneration (GBR) is a surgical procedure used to enhance bone growth of the alveolus for implant placement and around peri-implant defects through membranes with or without particulate bone graft or/and bone substitutes. Meanwhile, in guided tissue regeneration (GTR) occlusive barrier membranes are used for the treatment of periodontal bone defects, interfacing with gingival connective tissue/epithelium alveolar bone tissue to promote periodontal tissue regeneration [114].

A successful GBR is characterized by four principles: exclusion of epithelium and connective tissue from alveolar, space maintenance useful for an adequate regeneration, stability of the fibrin clot, and primary wound closure [115].

The process of regeneration in GBR depends on the migration of pluripotential and osteogenic cells to the bone defect site and contemporary by exclusion of cells impeding bone formation such as epithelial cells and fibroblasts. Thus, bioabsorbable or non-resorbable membrane are used in order to act as a barrier to prevent tissue invasion into the defect but at the same time to guide the regeneration process permitting bone augumentation.

Originally, membranes used in GBR were of expanded polytetrafluoroethylene (ePTFE). These membranes were developed in 1969 and they became the standard for bone regeneration [116]. They resulted biocompatible but non-resorbable and required a subsequently surgery after few weeks from treatment for their removal. So, because of this limitation, in addition to other undesirable characteristics, such as costs, patient discomfort and duration of the therapy, Titanium-reinforced PTFE were introduced as an alternative for ePTFE products. They provided advanced mechanical support which allows a larger space for bone and tissue re-growth permitting a correct rigidity, elasticity, stability and plasticity [117]. In the last decade, a number of resorbable membranes have been introduced. There are two categories of bioresorbable membranes: the natural and the synthetic membranes. Natural membranes are made of collagen, whereas synthetic products involve aliphatic polyesters such as polylactic acid (PLA), polyglycolic acid (PGA), Polycaprolactone (PCL) [118].

The great advantage of bioresorbable membranes is that they not need to be removal, have a greater cost-effectiveness and decrease patient morbidity. But, the main limitation of resorbable membranes is related to the resorption time, the degree of degradation and the effect of their degradation on tissue formation. The ideal membrane should be degraded or resorbed at the same rate of bone formation. It has been reported that an optimal stability of

membranes in vivo should be guaranteed for at least 4-6 weeks until several months, to allow successful regeneration [119].

4. Phytomedicine and bone

Natural medicine, known as phytomedicine, is the therapeutic use of products derived from natural sources, such as plants, animals or microorganisms and is considered one of the most attractive pharmaceutical research areas for the near future. Indeed, derivatives of natural origin have a predominant role in the evolution of medicine and nowadays represent an important resource of drugs [120]. In particular, polyphenols (PPH) are a large family of ubiquitous molecules mainly natural, but also synthetic/semisynthetic, organic chemicals derived from plants and marine organisms. These compounds, such as flavonoids, anthocyanins, phenolic acids, lignans and stilbenes, are complex structures which have in common the presence of benzenic cycles bearing one or several hydroxy functions. Several studies have showed an inverse correlation between the consumption of polyphenols and the risk of major illness such as cancer, cardiovascular diseases, type 2 diabetes mellitus, neurodegenerative diseases and osteoporosis [121] [122].

Curcumin is a phenolic product isolated from the rhizome of Curcuma Longa (turmeric), a perennial herb belonging to the ginger family, typically grown in south and southeast tropical Asia [123]. It is involved in a wide range of biological processes; numerous *in vitro* and *in vivo* studies have demonstrated that administration of this natural compound, exerts inhibitory effects on tumor progression, cell proliferation, metastasis. It is also involved in antioxidant and inflammatory pathway such as NF-kB, Akt, MAPK, p53, Nrf2, Notch-1, JAK/STAT, b-catenin, and AMPK [124], regulating important molecular targets. In addition, it has been shown that curcumin takes part in the regulation of bone remodeling, showing different effects on osteoblastic cell lines. Wen-Hsiung Chan *et al.* [125] in a study published in 2006 shown how different dosages of curcumin could influenced the cell death modes of Human Fetal Osteoblast cell line (HFOb 1.19). Indeed, curcumin exposure of MG-63 cells, a human osteosarcoma cell line, modulates osteoblastic differentiation through a mechanism partially

related to the inhibition of NO production [126]. Moreover, curcumin may promote osteoblast differentiation of rMSCs and inhibit adipocyte formation. The osteoblast differentiation results by an increase in ALP activity and the expressions of Runx2 and osteocalcin mRNA, while the decreased adipocyte differentiation is determined by the expressions of PPARγ2 and C/EBPα mRNA [127].

Recently, the use of curcumin in bone tissue has received attention due to its beneficial synergic effect with resveratrol. Together, these natural compounds, may be more effective than the individual compounds. Csaki C. *et al*. reported that inflammatory mediators, such as PGE2, leukotriene B4 (LTB4), COX2, MMP-1, MMP-3, and MMP-13 resulted down-regulated due to treatment of curcumin and resveratrol. Besides, their combination was able to prevent the activation of caspase 3, a protein affector in the apoptotic cells both by extrinsic and intrinsic pathways [128]. Thus, these studies have supported the potential role of their combination, suggesting how the use of this strategy *in vitro* may prevent not only the progress of a pro-inflammatory environment but also facilitate the bone regeneration.

Another compound considered as preventive nutraceutical compost because exhibits *in vitro* health-beneficial properties is quercetin [129]. It is a flavonoid, mainly presents as quercetin glycosides, most abundant in western diets. It is present widely in fruits and vegetables (apples, berries, onions, grapes, tea, tomatoes and red wine) as well as in some medicinal plants such as the perforate St John's-wort (*Hypericum perforatum*) and maidenhair tree (*Gingko biloba*) [130].

It shows potential pharmacological properties as anti-cancer and anti-inflammatory activities and also reduces the risk of cancer and cardiovascular disease. Its function is also linked to a relevant antioxidant activities and potential scavenger of free radicals [131].

Its effects on osteoblast function *in vitro* is conflicting. As reported by Kim *at al*. it promotes the proliferation, differentiation, and mineralization of osteoblasts with simultaneous

increasing in the production of osteoprogenitors [132]. On the contrary, it has also been shown to induce apoptosis in MC3T3-E1, cells murine calvarial osteoblast cell line [133].

Recent studies [134] have shown that 0.1 μM quercetin concentration does not show an effect on osteoblastic differentiation while higher values (10 μM) have a negative effect, reducing viability, expression of osteoblastic genes and mineralization, inducing the differentiation in adipocytes.

On the other hand, Swati Srivastava *et al.* [135] have reported that physiological doses of this compound may increase bone formation, promoting quercetin as a cure for osteodegenerative disorders. Indeed it increases proliferation and osteoblast differentiation of mesenchymal stem cells in a dose dependent manner, showing high expression of osteogenic marker genes and characteristic features of osteoblastic phenotypes. However, further research must be evaluated to better understand quercetin properties and its molecular mechanism in living cells.

4.1 Role of Resveratrol in bone regeneration

Resveratrol, chemically known as 3,5,4-trihydroxystilbene, is a naturally polyphenol produced by a wide variety of plants in response to injury, UV irradiation, ozone exposure and fungal attack [136].

It exists naturally as both cis- and trans-isomers and both isomers may have different biological effects. *Cis*-isomerization can also occur when the *trans* isoform is exposed to sunlight or to artificial or natural UV radiation at wavelengths of 254 nm or 366 nm. The trans isomer is the major and more stable natural form and most studies have used *trans* resveratrol for administration due to instability of the *cis* isomer [137] [138].

Resveratrol is the cause of the "French Paradox," the phenomenon by which a certain population of France (consumers of wine), in spite of a high fat diet and low exercise practice, appears to have less predisposition to heart diseases. This is explained by the crucial role in

cardiovascular protection provided by the phenolic content, mainly in resveratrol of grapes and wines [139]. It has antioxidant [140], anti-inflammatory and anticarcinogenic properties [141]. It is a cell cycle inhibitor [142], an anti-aging agent [141], neuroprotector [143], cardioprotector [144], and has great potential in the treatment of obesity and diabetes. Moreover, it is used to stabilize polyester films for packaging and potential biomedical applications.

Its beneficial effects have been supported by studies at cellular and molecular levels *in vitro* and *in vivo* models. This polyphenol can be considered as a dietary phytoestrogen with powerful beneficial effects on both estrogen receptors (ERs) expressing and non-expressing human tumors. Furthermore, some studies have suggested the use of resveratrol as valid alternative for therapy of osteoporosis [145]. The chemical structure of resveratrol is similar to that of the synthetic estrogen diethylstilbestrol (4,4' dihydroxytrans-diethylstilbene). It binds ERs in the low micromolar range with an affinity lower than that of estradiol; therefore, it behaves as a weak competitor. Despite the lower binding affinity, resveratrol may act as a superagonist in activating hormone receptor-mediated gene transcription [146] [147]. In particular, resveratrol activates the estrogen-mediated extracellular signal-regulated kinase (ERK) 1/2 signaling pathway regulating osteoblast differentiation and proliferation [12]. It is also considered a promising candidate for bone tissue engineering purposes because it has showed to stimulate bone cell proliferation and differentiation of MSCs (Figure 8). Its ability to promote their differentiation into osteoblasts is influenced by a specific concentration and seems to be caused by an effect dose-dependent as demonstrated by Lindsay Peltzas *et al.* [13].

Resveratrol can also significantly modulate osteogenic differentiation of undifferentiated cells involving specific molecular pathways such as NAD-dependent deacetylase sirtuin-1 (SIRT1) and Wnt signaling [7].

Zhou *et al.* [148] showed that resveratrol augmented Wnt signaling which stimulated osteoblastogenesis and bone formation. Treating human bone marrow-derived MSC with resveratrol promoted their differentiation toward osteoblasts by up-regulating Runx2 gene expression through the activation of Sirt1 [149].

In addition, epigenetic modifies should have an important role in regulating osteogenesis: the activation of Sirt1 in MSCs by resveratrol in addition with Runx2 acetylation/deacetylation influences their differentiation into osteoblasts. Shakibaei *et al*. have showed a study on MSCs and pre-osteoblastic cells treated with an osteogenic induction medium with or without the Sirt1 inhibitor nicotinamide and/or resveratrol. Osteogenesis resulted blocked by nicotinamide promoting adipogenic differentiation. But, in nicotinamide-treated cultures, pretreatment with resveratrol significantly enhanced osteogenesis by increasing expression of Runx2 [149].

Thus, based on these evidences, resveratrol offers a promising natural therapeutic agent for pathologies in which enhanced bone has required.

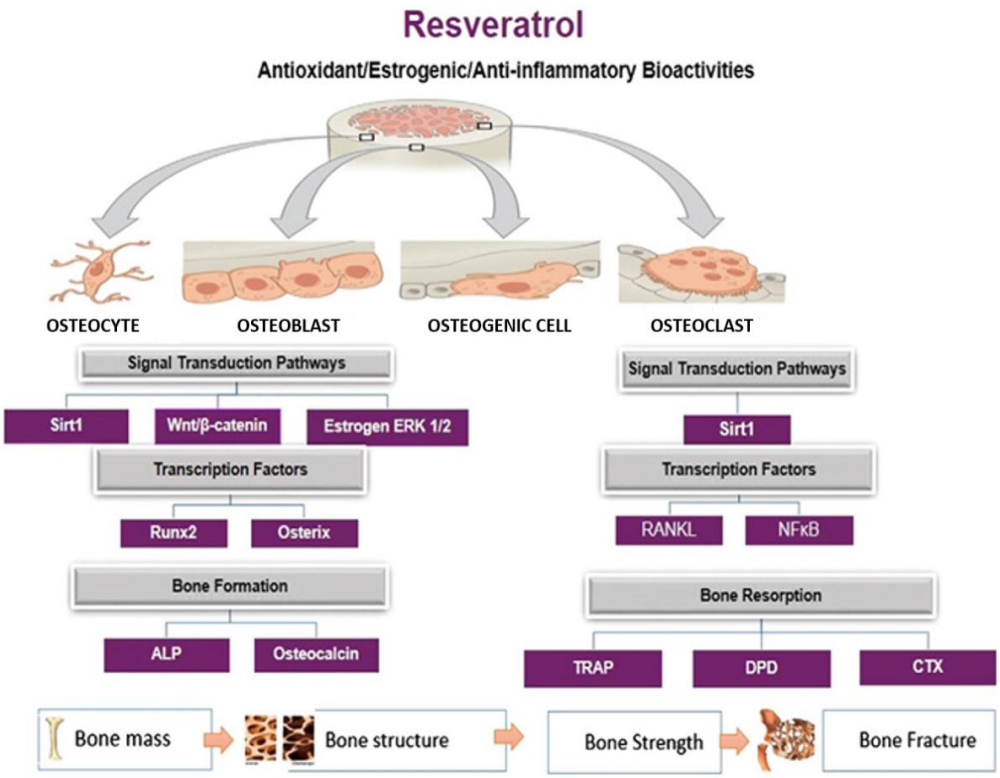

Figure 8. Schematic of potential molecular mechanisms of resveratrol on osteoblasts and osteoclasts. Abbreviations are ALP, alkaline phosphatase; CTX, C-terminal telopeptide of type I collagen; DPD, deoxypyridinoline; ERK, extracellular signal-regulated kinase 1/2; NFκB, nuclear factor kappa B; Runx2, runt-related transcription factor 2; Sirt1, Sirtuin1; TRAP, tartrate-resistant acid of the phosphatase; Wnt; canonical wingless/β-catenin signaling pathway.

Source: Janet C. Tou, Biochimica et Biophysica Acta, 2014

Materials and methods

5. Materials and methods

5.1 Electrospinning procedure

Poly(D L-lactide) (PLA, inherent viscosity: 0.55-0.75 dL g^{-1} in $CHCl_3$) and poly ε-caprolactone (PCL, inherent viscosity: 0.80 dL g^{-1} in $CHCl_3$) polymers were purchased from Lactel Absorbable Polymers (Pelham, AL).

Fibrous membranes of PCL and PLA alone or in presence of 1, 5 and 10 (wt %) of resveratrol (PCL-RSV or PLA-RSV) were prepared by electrospinning. Polyesters were dissolved in acetone (17.5 wt% for PCL; 12.5 wt% for PLA) and the RSV was slowly added to the polymer solution under vigorous stirring until complete dissolution.

Electrospinning of PCL, PLA, or PCL and PLA with RSV was carried out at room temperature at a constant voltage of 30 kV (HV Power Supply, Gamma High Voltage Research, Ormond, FL). A copper wire was mounted in the spinneret having an inner diameter of 0.8 mm and used as the positive electrode. Grounded aluminium foil was used as the counter electrode and mounted at a distance of 30 cm from the spinneret. Continuous PCL, PLA or PCL and PLA with RSV fibers were collected on the aluminium foil in the form of a fibrous mat. Electrospinning conditions were optimized to produce nano fibrous mats composed of individual fibers ranging from 0.3 to 0.9 μm in diameter and without bead formation. For convenience, the membranes obtained from PCL or PLA with RSV were hereafter defined as PCL-RSV*n* or PLA-RSV*n* where *n* is the amount of RSV present (worded as weight percentage).

5.2 Scanning Electron Microscopy (SEM) and fiber diameter

The morphology and the diameter of the electrospun nanofibers were analysed by a scanning electron microscope (SEM Mod. LEO 420, Assing, Italy). All samples were sputter coated with gold (Agar Automatic Sputter Coater Mod.B7341, Stansted, UK) at 40 mA for 180 s prior the analysis. The fibers diameter distribution was determined by Sigma SacnPro 5.

About 500 fibers were considered, taking their dimensions respect to the reference bar of SEM image.

Fiber diameter was determined by analyzing the SEM micrographs using the image software ImageJ 1.43u and employing its scale bar calibrated measurement function. For each membrane, 75 diameter measurements were taken and weighed by fiber length to determine the overall mean fiber diameter.

5.3 X-ray diffraction (XRD)

XRD data were collected using an automatic Bruker diffractometer (equipped with a continuous scan attachment and a proportional counter), with the nickel filtered Cu Kα radiation (λ = 1.54050 Å) and operating at 40 kV and 40 mA. The diffraction scans were recorded at 2θ = 2-40°, step scan 0.03° of 2θ and 3s of counting time.

5.4 Differential Scanning Calorimetry (DSC)

DSC measurements were carried out using a Mettler DSC 822/400 thermal analyzer instrument having sub-ambient capability. About 2-3 mg sample was placed in an aluminium pan and heated at a rate of 10 °C/min from 0 to 250 °C in a nitrogen atmosphere.

5.5 *Ex-vitro* degradation

The hydrolytic degradation tests were performed in a manner similar to the method used by Sen Gupta and Lopina [150] with same modifications. All nanofibrous membranes were circular punches in pieces of 15 mm, vacuum-dried for 24 hours and individually weighed. All samples were placed into individual vials and covered with aluminum foil to prevent drug degradation caused by light. Degradation kinetic was performed in:

1. Phosphate-buffered saline (PBS, 10 ml, pH.4) PBS contains 200 mg/L NaN_3 as a biocide;

2. Artificial saliva medium (SAGF, 10 mL pH. 6.8). SAGF was prepared from calculated amounts of chemicals supplied by Sigma-Aldrich according to the procedure described by Schiff et al. and Kocijan et al. [151] [152].

Solutions were renewed every 3 days and replaced with fresh solution. The samples were extracted at different time intervals (every day for 10 days, and then every week over a 60-day time period). At the time of extraction, samples (3 replicates) were removed from the incubator, gently washed with deionized water to remove latent salts, vacuum-dried at 40°C for 48 hours and weighed. Degradation (percent mass lost, w) was calculated using the following equation:

$$w = \left(\frac{w_1 - w_2}{w_1}\right) 100$$

where w_1 and w_2 are the dry mass of the sample before and after the hydrolytic degradation, respectively.

5.6 Drug release measurement

For drug release determination, samples were prepared as reported in *ex-vitro* degradation (paragraph 5.5). At predetermined time intervals, aliquots of 3 ml were taken from the tube and the medium was replenished by a fresh buffer. Resveratrol concentration was assayed using an high performance liquid chromatography with an automatic injector and a diode array UV–vis detector (HPLC-UV) according to Omar et al. [153] with some modifications. The analysis was carried out on Agilent 1260 Infinity Quaternary LC (Agilent Technologies, Santa Clara, CA, USA) equipped with a Diode-Array Detector (DAD). The chromatographic separation was performed on a Gemini® 5 µm C18 110 Å, LC Column 250 x 4.6 mm (Phenomenex, Torrance, CA, USA) protected by a guard column (Security Guard Cartridge C18, 4 × 2.0 mm inner diameter, Phenomenex, Torrance, CA) and maintained at 35°C. A linear elution gradient consisting of mobile phase A (0.1% acetic acid), B (Acetonitrile), and C (Methanol) was programmed as follows: initially 50% A, 45% B, and 5% C, linearly

changed to 30% A, 65% B, and 5% C over 5 min, and then held for 4 min at 30% A, 65% B, and 5% C. The system was then riequilibrated for 5 min with the initial solvent. The detection wavelength was set at 290 nm. The quantitation of resveratrol is by peak area ratio and is based on a standard curve in artificial saliva, generated by using an external standard. A linear curve is generated from a double analysis of six different standard concentrations. The resveratrol stock standard of 1 mg/ml was prepared in methanol, and subsequent dilutions were carried out to obtain six standard solutions (10, 20, 25, 30, 40, and 50 μg/mL). Additionally, six standard solutions (1, 2, 4, 6, 8, and 10 μg/ml) were obtained to determine the low concentrations of resveratrol. Prior to injection, the standards and samples were filtered through a 0.22 μm pore-size filter (Millipore, Bedford, USA). System control and data acquisition were performed using the ChemStation software (Agilent Technologies).

5.7 Cell isolation and characterization

Human Dental Pulp Stem Cells (DPSCs) were isolated from normal, non-carious impacted third molars from 10 adults (18–22 years of age) as previously described [154]. The teeth were obtained in compliance with Italian legislation (including informed consent and Institutional Review Board approval of the protocol number 7413). The teeth were cleansed of external organic and inorganic debris with 70% isopropanol. In cases where the teeth were incompletely developed, the apical papilla was removed to prevent contamination [155]. Teeth were sectioned longitudinally; pulpal tissue was gently removed with tissue forceps and cut into small fragments. The tissue was then placed in PBS prior to enzymatic digestion with 3 mg/ml of collagenase type I and 4 mg/ml dispase for 30 min at 37°C. The digested mixtures were passed through a 70-mm cell strainer (Falcon, Italy) to obtain single-cell suspensions and centrifuged for 10 minutes at 1,800 rpm. Cells were seeded onto six-well plates and cultured in Growth medium [GM, α-minimum essential medium (α-MEM) supplemented with 15% FBS, 2 mM L-glutamine, 100 mM L-ascorbic acid-2-phospate, 100 U/ml penicillin-G,

100 mg/ml streptomycin, and 0.25 mg/ml fungizone (Hyclone, Italy)] and maintained in 5% CO_2 at 37°C.

DPSCs multipotency and differentiation capacities was confirmed by Reverse Transcription-polymerase chain reaction (RT-PCR). For osteogenic differentiation, cells were cultured at 5 x 10^3 cells/well in 6-well plates until 50% of confluence. Then the growth medium was replaced with osteogenic medium that consisted of α-MEM supplemented with 0.2 mM L-ascorbic acid 2-phosphate (Sigma, USA), 0.01 μM dexamethasone (Sigma, USA), and 10 mM β-glycerol phosphate (Sigma, USA). To verify adipogenic differentiation, the cells were cultured at 5 x 10^3 cells/well in 6-well plates and allowed to grow to 50% confluency. The medium was then replaced with adipogenic medium that consisted of α-MEM supplemented with 1 mM dexamethasone and 60 mM indomethacin (Sigma, USA). For chondrogenic differentiation, cells were placed in a conical tube, pelleted by 400 g centrifugation, provided with chondrogenic medium, and incubated in an atmosphere of 5% CO_2 and 37˚C. The chondrogenic medium consisted of hMSC Chondrogenic SingleQuots (Lonza Walkersville, Inc., USA) supplemented with 10 ng/ml TGF-β3 (Peprotech, UK). The cultures were maintained for 3 weeks with twice weekly medium changes. At the end of the maintenance period, total RNA was extracted as reported in Real-time PCR paragraph (paragraph 5.11).

5.8 Colony-forming ability and proliferation of DPSCs

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests progenitor cells ability to undergo "unlimited" division, to differentiate and proliferate.

Colony Forming Unit-Fibroblast (CFU-F) assays were performed by plating third to sixth passage DPSCs isolated from 10 donors in a 6-well plate at 50 and 100 cells/well in GM. After 14 days in culture at 37°C in a humidified atmosphere containing 5% CO_2, the cells

were washed twice with PBS, fixed with ice-cold methanol for 5 min at room temperature, and stained with 0.3% crystal violet for 15 min. The cells were washed with distilled water and the number of colonies was counted. Colonies greater than 2 mm in diameter were enumerated.

Proliferation assay was performed by seeding DPSCs in a 6-well plate at 4 x 10^3 cells/well in duplicate, and incubating them in GM for 14 days at 37°C in a humidified atmosphere containing 5% CO_2. At days 3, 6, 9 and 12, the cells were harvested by trypsin-EDTA treatment, washed in GM and centrifugated at 1,500 rpm for 10 min. The pelleted cells were then counted with a hemocytometer and their viability determined by the trypan blue dye exclusion test.

5.9 Magnetic-activated cell sorting (MACS)

To obtain STRO-1^+ stem cells, DPSCs were directly sorted from pulp cell cultures at passage 3 with immunomagnetic beads Dynabeads according to the manufacturer's protocol (Life Technologies, Milan, Italy). In particular, approximately 5 × 10^6 cells were incubated with mouse anti-human STRO-1 at 4°C for 30 minutes, washed with PBS/5% bovine serum albumin (BSA), and resuspended with rat anti-mouse IgM-conjugated Dynabeads on a rotary mixer for 60 minutes. After washing, bead-positive cells were separated with a magnetic particle separator and subsequently placed into 75 cm^2 culture flasks. Immunosorted DPSCs were cultured, passaged in the routine culture media (α-MEM) at 37°C in 5% CO_2, and observed under the phase-contrast inverted microscope (Olympus). Approx 5% of DPSCs in the primary cells can be harvested by STRO-1-mediated MACS method.

After cell sorting, each of the following experiments was performed in triplicate on pooled STRO-1–sorted cells (STRO-1^+ cells).

5.10 Alkaline Phosphatase Activity

Alkaline Phosphatase Activity (ALP) is a typical marker for early osteoblastic differentiation. ALP activity was assessed as reported by Wang et al. [156] on DPSCs cells cultured for 1, 7, 14, and 28 days in GM and osteogenic-induction medium (OIM, α-MEM, 10% FBS, 100 nM dexamethasone, 10 mM sodium β-glycerophosphate, 100 mM L-ascorbic-acid-2-phosphate, 2 mM L-glutamine, 100 U/ml penicillin-G, 100 mg/ml streptomycin, and 0.25 mg/ml fungizone). At predetermined days, the cells were scraped into cold PBS, sonicated in an ice bath and centrifuged at 1500 g for 15 min. ALP activity was measured in the supernatant using p-nitrophenyl phosphate as a phosphatase substrate. The absorbance was measured at 405 nm recording data every 5 min for 30 to 60 min. For end-point the reaction were incubated for 30-60 min with 50 µL of Stop Solution into each well before reading. The amount of ALP in the cells was normalized against total protein content.

5.11 Osteogenic-related gene expression by real-time polymerase chain reaction

Real-time Polymerase Chain Reaction (RT-PCR) permits simultaneous amplification and detection of specific DNA-sequences. The amount of product formed was monitored during the course of the reaction through the fluorescence of the probes that was introduced into the mix. The number of amplification cycles required to obtain a particular amount of DNA molecules was registered [157].

To evaluate the expression levels of osteontogenic-related genes corresponding RNA were quantified in form of cDNA. Indeed, total RNA was extracted from cells seeded in the presence of membranes for 1, 3, 7, 21 days, using TRIzol reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. Total RNA (0.2 µg) was first treated at 37°C for 30 min with DNase (Promega, Milan, Italy) and then subjected to reverse transcription (RT) with 0.4 µg random hexamers and 20 U AMV reverse transcriptase (Promega) in a 25-µL

reaction mixture at 42 °C for 1 h. The resulting cDNA mixture was amplified by real-time polymerase chain reaction (PCR) using specific primers listed in Table 3.

Gene	Accession no.	Forward primer	Reverse primer
Receptor activator of nuclear factor kappa-B ligand (RANKL)	AF019047	TGATTCATGTAGGAGAATTAAACAGG	GATGTGCTGTGATCCAACGA
Runx 2 (*RUNX2*)	NM_001024630	ACCGTCTTCACAAATCCTCCC	CTGTCTGTGCCTTCTGGGTT
Osteocalcin (*OCN*)	NM_199173	ATTGTGGCTCACCCTCCATC	GTAGGCCAAACCCCAAAGGA
Osteonectin (*ONN*)	NM_003118	GGGCTTCTCCTCCTCTGTCT	AACCGATTCACCAACTCCAC
Osteopontin (*OPN*)	NM_001040058	GCCGAGGTGATAGTGTGGTT	CATTCAACTCCTCGCTTTCC
Bone sialoprotein (*BSP*)	NM_000582	CAGGACTGCCAGAGGGTAAG	TTCAAAGCCAAGTTCAGAGATGT
Sp7 transcription factor (*OSX*)	AF477981	GTGGAACAGGAGTGGAGCTG	TCCTCTCTGGAGGTCTGGC
Hypoxanthine phospho-ribosyltransferase 1 (*HPRT1*)	NM_000194	TCCATTCCTATGACTGTAG	ATTATACTGCCTGACCAA
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	NM_002046	GGAGTCAACGGATTTGGTCGT	ACGGTGCCATGGAATTTGC
Cathepsin K (CTSK)	NM_000396.3	GTTGTATGTATAACGCCACGG	CTTTCTCGTTCCCCACAGGA

Table 3. Specific primers used in RT-PCR

Real-time PCR assays were run on an Opticon-4 machine (Bio-Rad, Milan, Italy). The reactions were performed using 25µl of SYBR Green PCR Master mix per reaction (Invitrogen). The PCR conditions were as follows: AmpliTaq Gold DNA Polymerase (Life

Technologies) activation for 10 min at 95 °C and 40 cycles at 95 °C (denaturation) for 15 s and 60 °C (annealing/extension) for 1 min. All reactions were run in triplicate and were normalized to the house-keeping genes, *HPRT1* and *GAPDH*. Relative differences in the PCR results were calculated using the comparative cycle threshold (CT) method. The variations in gene expression are given as arbitrary units.

5.12 Resveratrol concentration on RANKL-induced osteoclast formation

Osteoclast precursor (OC) (Lonza, Milan) were seeded on a 24-well plate at a density of 1.25 x 10^4 cells per well. These cells were cultured in presence of different concentration of resveratrol (0, 0.5, 1, 2,5, and 5 μM) or PCL, PLA, PCL-RSV, PLA-RSV for 3-14 days with 3 ng/ml of recombinant mouse RANKL and stained for TRAP activity using the Leukocyte Acid Phosphatase kit (Sigma–Aldrich) following the instructions from the manufacture. TRACP-positive multinucleated cells having three or more nuclei were considered as osteoclasts, and their number was counted in randomly selected fields in different areas of each well.

5.13 Statistical Analysis

All quantitative data are presented as the mean ± SD. Each experiment was performed at least 3 times. Student's t test was used for the resveratrol release. Statistical analyses for the cell migration assay, and quantitative real-time PCR were performed by 1-way analysis of variance (ANOVA) with Bonferroni's post hoc test.

Results and Discussion

6. Results and Discussion

6.1 Physico-Chemical characterization of PCL-RSV and PLA-RSV nanofiber membranes

Electrospinning is a polymer processing technique that has great potential in the field of regenerative medicine because of the generation of non-woven fibrous scaffolds with fiber diameter sizes ranging on the order of nanometers to microns. Electrospun fibers are ideal for the local delivery of bioactive molecules because of their high surface area to volume ratio. Maintaining the bioactivity of the compounds during the electrospinning process and incorporating high amounts of active substances in the electrospun fibers have been active areas of research.

Recently, electrospinning has received considerable attention as an alternative approach for the fabrication of fibrous scaffolds with excellent biocompatibility and biodegradability, to guide tissue regeneration and to incorporate bioactive molecules such as drugs, proteins, and genes [158] [159] [160] [161]. To date, a range of polymers, including polyglycolic acid (PGA), polycaprolactone (PCL), polylactic acid (PLA), and their copolymers, have been developed as scaffolds by electrospinning for regenerative medicine applications due to their superior mechanical properties, spinnability, flexibility, biocompatibility, porosity, and controllable degradability [162] [163].

PCL-RSV and PLA-RSV nanofiber membranes were prepared by electrospinning PCL or PLA. Within the electrospinnng process, concentration of the spinning dope, flow rate of the spinning dope, spinning voltage, fiber collect distance and other factors will affect the diameter of the nanofiber. In figure 9 was reported the electrospinning apparatus used for the membranes preparation.

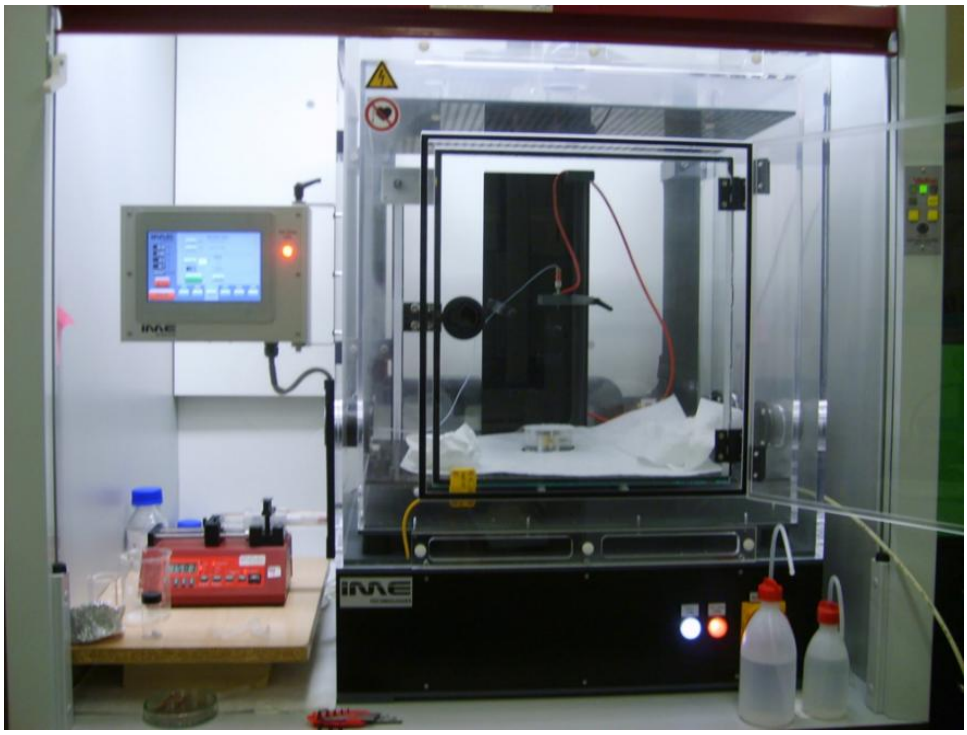

Figure 9. Electrospinning apparatus set-up used for membrane preparation

The detailed electrospinning conditions were reported in Table 4.

Polymers concentration (w/v)%	Nanofiber collect distance (cm)	Spinning voltage (kV)	RSV concentration (mg/ml polymers solution)
12.5	15	30	1.0
			5.0
			10.0
12.5	20	30	1.0
			5.0
			10.0
15.0	15	20	1.0
			5.0
			10.0
15.0	20	30	1.0
			5.0
			10.0
17.5	20	25	1.0
			5.0
			10.0
17.5	30	30	1.0
			5.0
			10.0

Table 4. Electrospinning conditions of nanofibers spun

The electrospinning process can be influenced by many interrelated internal as well as external parameters that, as a consequence, could prejudice the fibers morphology. For this reason, a trial-and-error approach was typically employed by varying the solution properties, the spinning parameters, and polymers concentration until uniform defect-free fibers were obtained.

According to Tammaro et al. [164, 165] acetone was chosen as solvent for PCL, PLA and PCL or PLA loaded with RSV. The mixtures were electrospun using the selected processing

parameters: voltage 30 kV, needle-collector distance 30 cm, flow rate 4 mL/h, needle diameter 0.8 mm, and polymers concentration of 17.5 for PCL and 12.5 for PLA. Among the RSV considered concentrations (1, 5, and 10 mg/mL polymer solution), 10 mg/mL was found to be optimal, therefore PCL-RSV_{10} and PLA-RSV_{10} were used for the further analyses.

Basically, a scaffold should be porous in order to permit the infiltration of cells, oxygen and nutrients through it, thereby promoting cell proliferation. Thus, the fabricated nanofibers having highly interconnected pores with suitable morphology for cell diffusion and propagation can be used as a wound dressing material. The morphology of the electrospun membranes was characterized via scanning electron microscope (SEM). SEM morphological analysis demonstrated that both PCL-RSV_{10} and PLA-RSV_{10} membranes had a three-dimensional interconnected pore structure with randomly oriented nanofibers (Figure 10). The pure PCL fibers, shown in figure 10A are very smooth and flat, while when RSV was added the fibers surface becomes increasingly roughened showing wrinkles along the fibers (Figure 10B). The same characteristics were observed in PLA-RSV fibers (Figure 10D). Moreover, no drug particles were observed on the surface of both fibers, demonstrating excellent compatibility between the compound and the polymers.

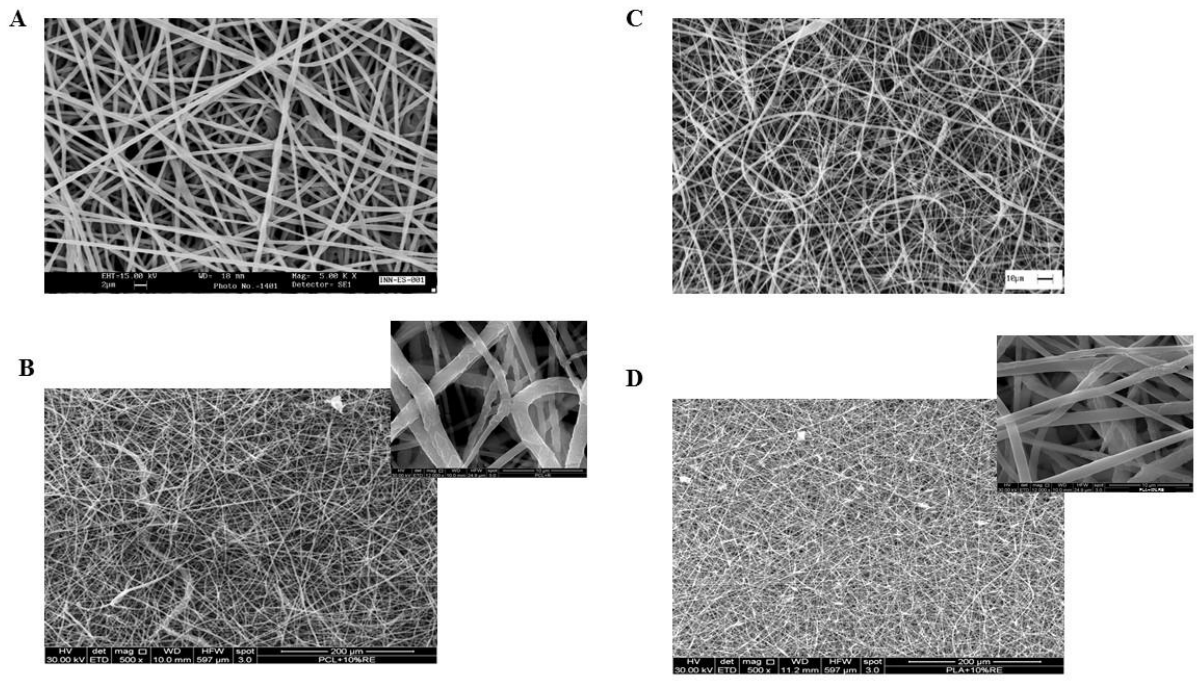

Figure 10. SEM images of PCL (A), PCL-RSV$_{10}$ (B), PLA (C) and PLA-RSV$_{10}$ (D) electrospun membranes.

The average fiber diameter of membranes was found to range between 0.60 microns to 0.95 microns in diameter for PCL-RSV$_{10}$ and from 0.30 microns to 0.65 microns in diameter for PLA-RSV$_{10}$ appearing almost similar to that of pure PCL and PLA equivalents. As reported in figure 11, nanofibers displayed random orientation and relatively uniform distribution of fiber diameter.

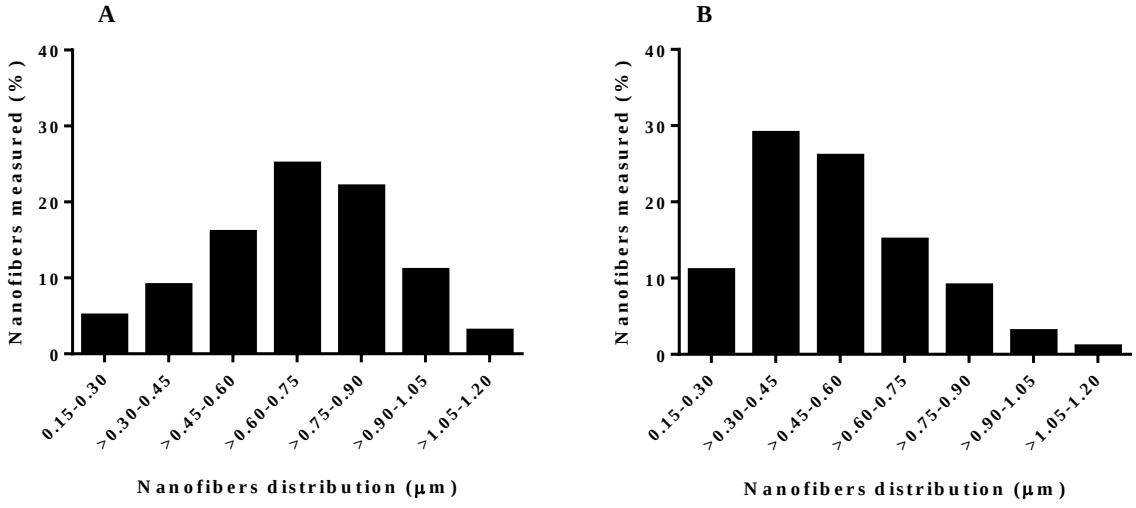

Figure 11. Nanofiber diameter size distribution of PCL-RSV$_{10}$ and PLA-RSV$_{10}$ membranes

The physicochemical properties of PCL-RSV_{10} and PLA-RSV_{10} membranes were characterized by X-ray diffraction, DSC, RSV release and degradation profiles.

XRD patterns of PCL spun mats show a diffraction peak typical of a semicrystalline polymer, with two sharp and intense crystalline peaks observed at 2θ values around 21.6° and 23.3°, and a broad shoulder at 22.2° [166] [167]. This pattern is due to (110), (200), and (111) planes, respectively, of an orthorhombic crystalline structure of PCL (Figure 12).

Doping PCL with resveratrol did not bring about significant changes in the polymer spectral pattern, while additional small peaks due to resveratrol were visible at 2θ values of 6.6, 19.2, and 28.4 (Figure 12A).

This outcome demonstrated that most of resveratrol is amorphized during the spinning process, and only a small fraction was able to crystallize within the polymer matrix. Amorphization of resveratrol possibly improves the dissolution of the additive, which, in turn, increases the drug delivery potential of the PCL-based spun mats [168] [169].

As for PLA, neat polymer did not show any evidences of crystalline peaks, demonstrating the amorphous nature of the spun fibers. Moreover, no crystalline reflexes of resveratrol were noted, suggesting that the additive embedded in the polymer matrix was completely amorphous (Figure 12B).

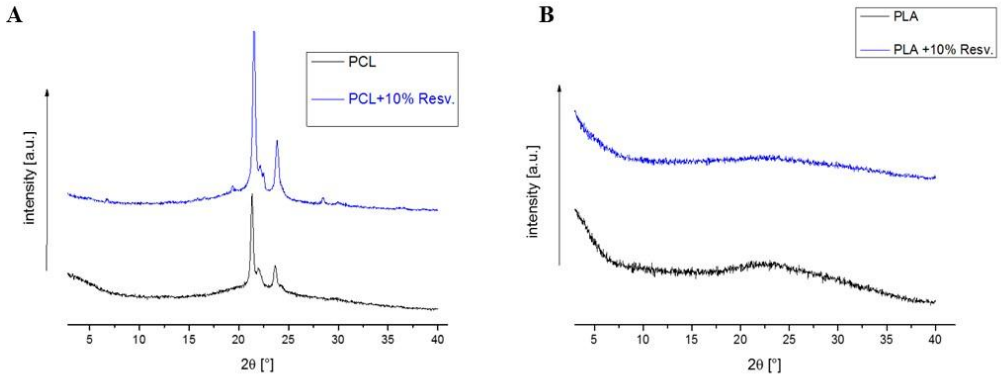

Figure 12. X-ray diffraction spectra of PCL, PCL-RSV_{10} (A), PLA, and PLA-RSV_{10} (B), confirming amorphization of RSV

XRD results were confirmed by DSC characterization. The thermogram related to PCL-RSV_{10} membrane showed the typical melting signal of this polymer, peaked at about 56°C (Figure 13A). It is also worth noting that spinning also caused amorphization of resveratrol, since no peaks due to its melting could be noticed in the range of 250-265°C, which is the expected temperature range of resveratrol melting. The thermogram of PLA-RSV_{10} only showed the glass transition signal of the polymer, at about 60°C (Figure 13B).

In this case, no melting peak was detected, confirming the amorphous nature of the polymer.

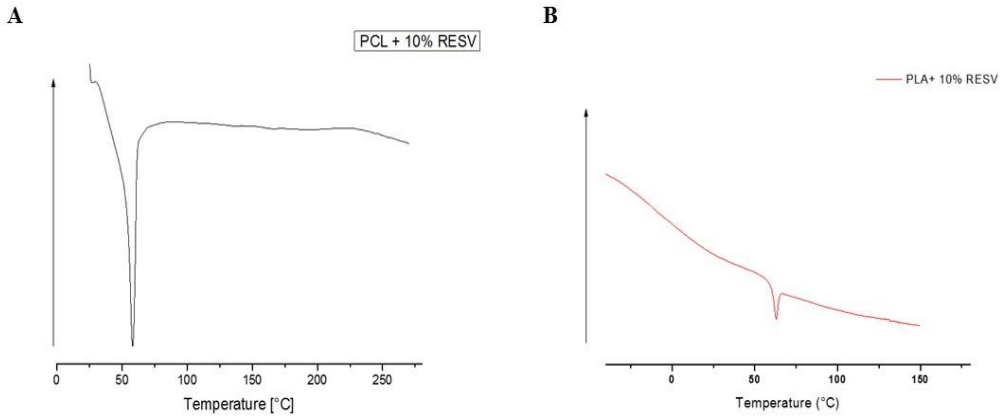

Figure 13. DSC curves of (A) PCL-RSV_{10} and (B) PLA-RSV_{10}

6.2 Degradation rate

In tissue regeneration applications, a controlled rate of scaffold degradation permits an appropriate cellular reorganization and tissue remodeling. Therefore, understanding the effects of biological fluids such as saliva on scaffold degradation is needed.

PCL is degraded by hydrolysis of its ester linkages in physiological conditions (such as in the human body) and has therefore received a great deal of attention in order to be used as an implantable biomaterial. PCL undergoes a two-stage degradation process: firstly, the non-enzymatic hydrolytic cleavage of ester groups and secondly, when the polymer is more highly crystalline and has a low molecular weight (less than 3000) the polymer is shown to undergo intracellular degradation as this was observed during experiments of PCL fragments uptake in

phagosomes of macrophages and giant cells and within fibroblasts [170]. This supports the theory that PCL may be completely resorbed and degraded via an intracellular mechanism once the molecular weight was reduced to 3000 or less. It was also noted that in the first stage the degradation rate of PCL is essentially identical to the *in vitro* hydrolysis at 40°C and obeyed first-order kinetics. It was concluded that the mechanism of PCL degradation could be attributed to random hydrolytic chain scission of the ester linkages, which caused a decrease in molecular weight [171].

PLA is a biodegradable polymer, which is naturally hydrophilic due to its polar oxygen linkages. It contains a methyl side group, which confers hydrophobic properties to this polymer [110]. The natural hydrophilic characteristic of PLA is responsible for its moderate decomposition by the surrounding moisture and temperature. The first stage of PLA degradation is usually the reduction of its molecular weight by hydrolysis to <10 kDa before it becomes biodegradable. The hydrolysis of PLA occurs by random cleavage of the –C–O– ester bond by water molecules. The hydrolysis products, which may contain fragments of lactic acid, oligomers, and other water soluble products, can then be consumed by microorganisms to produce carbon dioxide (CO_2), water (H_2O) and solid biomass. This reaction can be increased under acidic or basic conditions or in the presence of high moisture and high temperature [111]. Since hydrolysis of PLA is influenced by ambient moisture and temperature, it is possible to accelerate the diffusion of water and hence increases the hydrolysis or degradation of this polymer by subjecting it to thermophilic temperatures above 50°C.

In drug-loaded nanofibers, the degradation rate of the polymer may also influence the release rate of the drugs. However, the desired degradation rate of the polymers will depend on the intended application and performance of the implant which may range from weeks to years.

An *in vitro* degradation study is carried out by measuring the weights of a sample before and after treatment in a digesting solution. The *in vitro* degradation profiles of PCL-RSV_{10} and PLA-RSV_{10} membranes showed the stepwise patterns (Figure 14). In the first step, for the period up to 1 week of incubation, PLA-RSV_{10} membrane exhibited a very fast weight loss rate (about 4% loss of the initial weight), while PCL-RSV_{10} presented a relative weight loss rate (about 1% loss of the initial weight). After that period, the mass loss was decelerated for both membranes until reaching the plateau at two weeks or 4 weeks. In the second step, the degradation was reaccelerated, and additional weight loss of more than 8% was observed in PLA-RSV_{10} membranes until eight weeks. Different from that of RSV-loaded membranes, the mass loss of pure PCL and PLA equivalents was insignificant during test periods.

The differences between the two membranes might be attributed to the difference of hydrophilicity of PCL respect to PLA electrospun membranes.

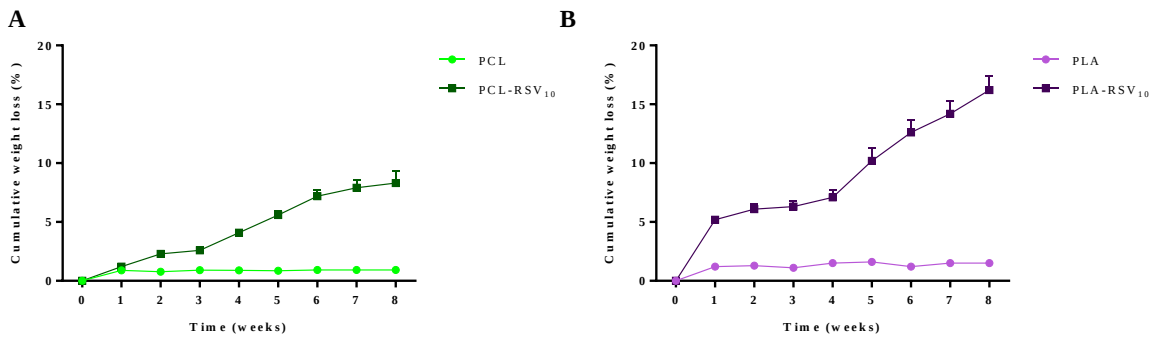

Figure 14. *In vitro* degradation profiles of (A) PCL, PCL-RSV_{10} and (B) PLA and PLA-RSV_{10} nanofibers membrane

5.3 Resveratrol Release

Shrikar et al. reported that the dominated mechanism of drug release from polyesters nanofibers is desorption of the embedded compound through nanopores in the fibers or from

the outer surface of the fibers in contact with the medium. A practically important outcome of this surface desorption mechanism is that only the compound on the surface and in pores can be released, whereas the drug loaded between polymer chains cannot be released with the same kinetics scales [172]. Most likely the water did not have access to dissolve the drug captured between polymers chains with bi-phase release kinetic: rapid drug release in the first 24 h, followed by a prolonged release.

As reported in figure 15, the release of resveratrol from PCL-RSV$_{10}$ showed a burst effect less as compared with PLA-RSV$_{10}$. Sustained release of resveratrol from PCL-RSV$_{10}$ was observed until 30 days, with the total amount of resveratrol released at the end of analysis being 32 %, whereas PLA-RSV showed a cumulative release of 44 % at the end of 30 days.

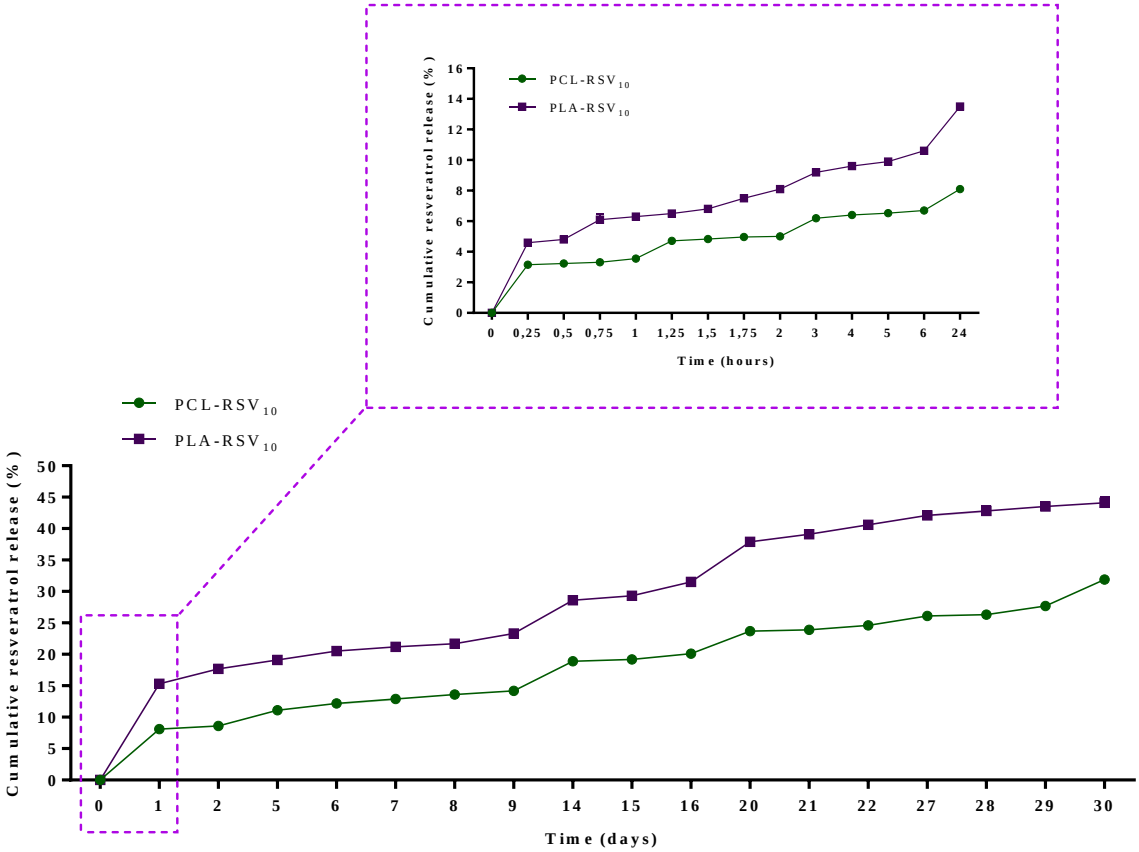

Figure 15. Release profile of resveratrol from PCL-RSV$_{10}$ and PLA-RSV$_{10}$ nanofibers membrane during 30 days

6.3 DPSCs Characterization

Many studies have demonstrated that Dental Pulp Stem Cells (DPSCs) have the ability of both self-renewal and multilineage differentiation into the neurogenic, osteogenic, dentinogenic, and myogenic cell lineages when cultured in different inductive media [173]. For the lack of specific cell surface markers, the identification of DPSCs mainly relies on their biological features, including small cell volume, high proliferation potency, high clonogenicity, self-renewal, and multiple differentiation potential [174] [173]. However, significant variability in growth patterns, CFU-F efficiency and stem cell immunophenotypic profiles have been recorded in DPSC cultures established from different healthy donors of similar age and stage of third molar root development [175].

To identify the self-renewal potential of isolated cells of 10 donors, the ability of colony-forming unit-fibroblast (CFU-F) formation and their proliferation profiles were determined (Figure 16B). Figure 16A showed, just as an example, the number of CFU-F of 3 donors demonstrating that more than 80% of isolated cells were capable of forming colonies when seeded at low cell density. In addition, the cell proliferation of isolated DPSCs increased in a time-dependent manner (Figure 16B). The results demonstrated that cells isolated from all donors exhibited a similar CFU-F and proliferation profile, therefore each of the following experiments was performed in triplicate on pooled cells.

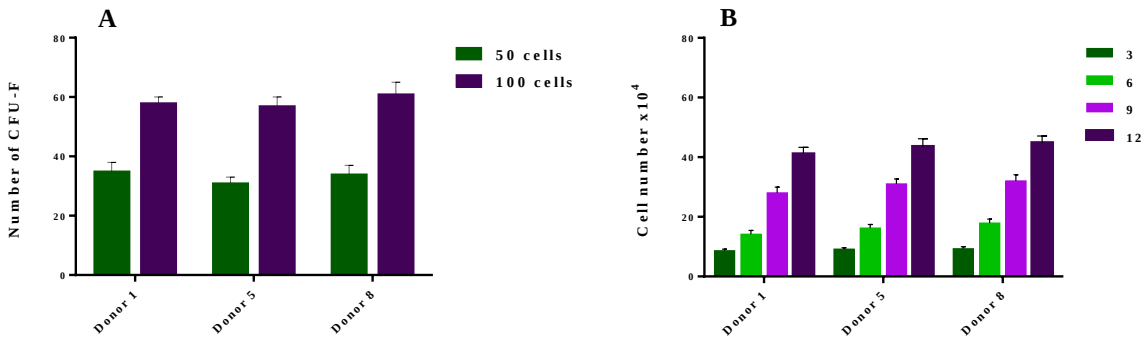

Figure 16. (a) The incidence of colony-forming cells from dental pulp cells of 3 selected donors at various plating densities. (b) Proliferation rate after 3, 6, 9 and 12 days of culture of 3 selected donors. The bars represent means ± SD for three experiments each performed in triplicate

DPSCs *in vivo* usually remain quiescent within adult dental pulps, but respond during injury to produce progenies with high proliferative potential which can differentiate into terminally differentiated odontoblasts. Thus, the amount of DPSCs in the normal dental pulp remains relatively constant. These lineage-specific progenies *in vivo* may bring about different cell types which contribute to the maintenance and homeostasis of dental pulp tissues. The typical surface markers of mesenchymal stem cells are CD44, CD73, CD90, CD105, CD271 and STRO-1, while the negative markers are CD34, CD45, and HLA-DR [176]. However, there is no specific, strict marker characterizing DPSCs, which are considered a heterogeneous population. Indeed, DPSCs acquired by single colony-derived methods cannot provide a cell population with standardized differentiation potential to be used in tissue engineering [154].

For this reason, different mesenchymal stem cell markers were used to select different subsets of DPSCs displaying different biological behaviors [177]. Magnetic-activated cell sorting (MACS) with antibodies against specific cell receptors, such as STRO-1, can form a more standardized way of retrieving and culturing DPSCs. Previous studies demonstrated that DPSCs contained a STRO-1-positive (STRO-1^{+}) cell population [174] identifying a subgroup of cells with odontogenic and osteogenic properties [178] [179]. These sorted stem cells

(STRO-1$^+$) in high purity might provide a better cell source for therapeutic purposes than heterogeneous unsorted cells. Because of this, cells with pronounced expression of stem cell properties, including high growth potential, and high clonogenicity (CFU-F efficiency > 35%) were sorted by MACS with STRO-1 antibody. The amount of sorted stem cells (STRO-1$^+$ cells) ranged from 9 ± 2.5% to 14 ± 3.7%.

During their differentiation, osteoblasts produce alkaline phosphatase (ALP), type I collagen, and osteopontin, which were associated with matrix maturation and mineralization [180]. Since ALP is a well-recognized early marker of osteogenic differentiation, ALP activity was measured in both DPSCs and STRO-1$^+$ cells cultured in osteogenic-induction medium. Cells cultured on growth medium was used as control (CTL). Figure 17 showed that the ALP activity gradually increased for 28 days in cells grown in osteogenic-induction medium respect to cells cultured onto growth medium ($p < 0.01$). Moreover, ALP activity of STRO-1$^+$ cells increased ($p < 0.05$) compared to that of DPSCs.

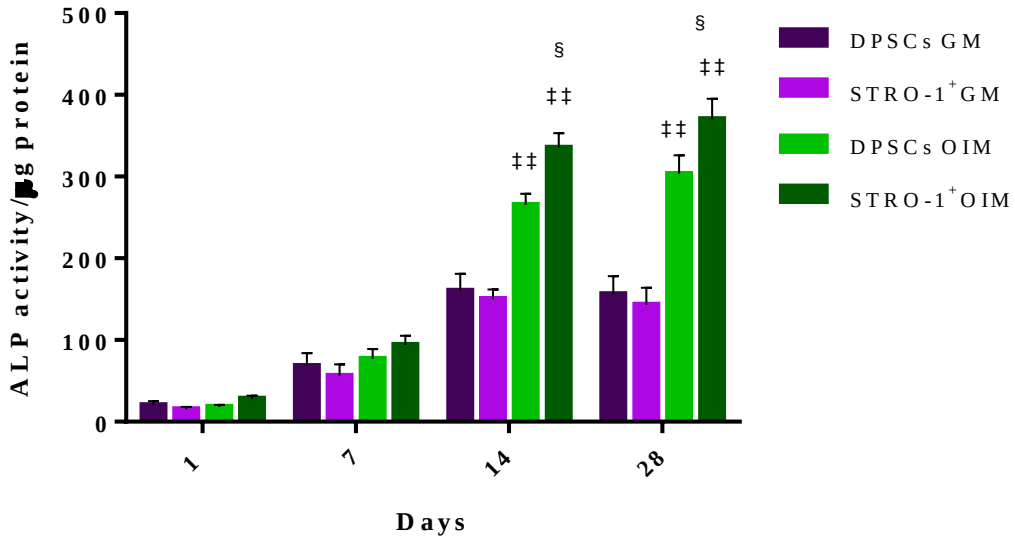

Figure 17. ALP activity of DPSCs and STRO-1$^+$ cells cultured in growth (GM) and osteogenic-induction medium (OIM). The bars represent means ± SD for three experiments each performed in triplicate. Statistically significant difference ‡‡ $p < 0.01$ *versus* DPSCs GM and STRO-1$^+$ GM; § $p < 0.05$ *versus* DPSCs OIM and STRO-1$^+$ OIM

6.4 Resveratrol promotes DPSCs differentiation

Several studies have been reported the effect of resveratrol as a stimulator of osteogenesis in human mesenchymal stem cells (hMSCs), human adipose derived stem cells (hADSCs), and pre-osteoblastic MC3T3-E1 cells demonstrating its effect on proliferation and osteogenesis in a dose-dependent manner [3]. hADSCs cultured with 12.5 μM, 25 μM, and 50 μM resveratrol showed the highest proliferation rate when exposed to 12.5 μM resveratrol, and the highest levels of ALP when cultured with 25 μM resveratrol. Doses of 50 μM resulted in extremely low cell numbers and ALP production. Another study concluded that doses higher than 25 μM of resveratrol are potentially cytotoxic, and that 12.5 μM resveratrol results in the greatest mineralized matrix after 4 weeks *in vivo* [181]. In addition, hMSCs cultured with varying doses of resveratrol produced the highest calcium deposition and greatest proliferative capabilities when exposed to a concentration of 10 μM [182] [12].

To analyze the role of resveratrol in promoting DPSCs differentiation towards osteoblast-like cells, ALP activity was measured in STRO-1^+ cells cultured for 28 days in GM with or without different concentration of RSV (1-25 μM). Cells cultured for 28 in OIM were used as control. It is important to note that in all studies, STRO-1^+ cells exhibited a normal proliferation curve with no significant difference in cell growth between the study groups (data not shown).

As reported in figure 18, resveratrol treatment of STRO-1^+ cells in the range from 1 μM to 15 μM resulted in a dose-dependent increase in ALP activity. However, resveratrol at a higher concentration (25 μM) led to little decrease in ALP activity when compared to control. These results confirmed that resveratrol is able to induce commitment toward an osteogenic lineage at concentration lower to 25 μM.

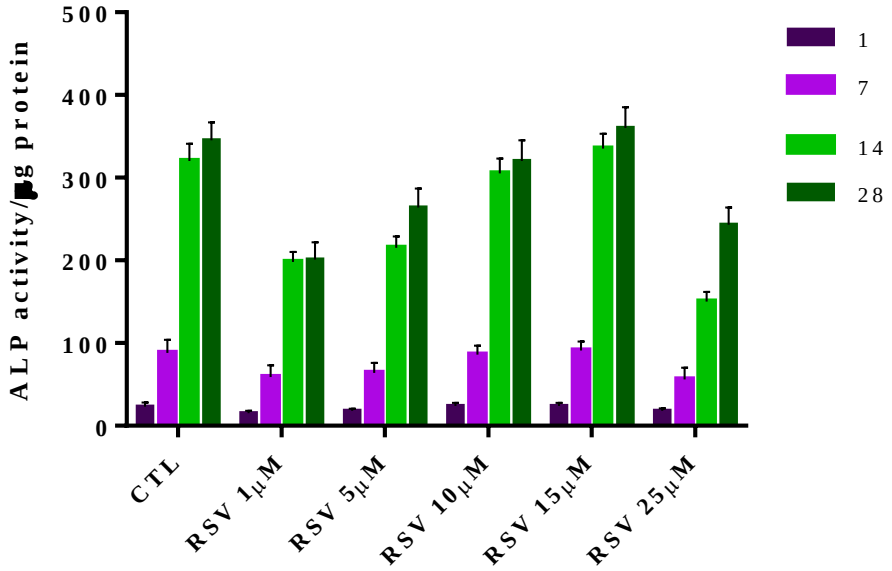

Figure 18. ALP activity of STRO-1^{+} cells cultured in GM in presence of different concentrations of resveratrol (1 µM to 25 µM). Cells cultured in OIM were used as control (CTL). The bars represent means ± SD for three experiments each performed in triplicate

Osteogenesis is a strictly controlled developmental process in which numerous extrinsic factors, including hormones and growth factors, activate osteoblast-specific signaling proteins and transcription factors required for osteoblast differentiation [183].

Several studies in humans and mice have identified two transcriptional factors that regulated the early stages of stem cells differentiation along the osteoblast lineage: *Runt-related transcription factor 2* (*RUNX2,* also called core binding factor alpha 1 - *Cbfa1*) and *Osterix* (*OSX*). RUNX2 regulates the expression of several important osteoblast proteins, including *osteopontin* (*OPN*), *bone sialoprotein* (*BSP*) *type I collagen*, *osteocalcin* (*OCN*), and *osteonectin* (*ONN*).

Once Runx2 is activated, the cells undergo a 3-stage differentiation [184]:

- In Stage 1 the cells continue to proliferate and express fibronectin, collagen, and TGFβ receptor 1.

- In Stage 2 they exit the cell cycle and start differentiating, while maturating the extracellular matrix with ALP and collagen.
- In Stage 3 matrix mineralization occurs when the organic scaffold is enriched with osteocalcin, which promotes deposition of mineral substance [185].

To determine whether resveratrol released from electrospun membranes could drive the expression of selected osteoblast marker genes in STRO-1^+ cells, mRNA levels of the early-stage markers *RUNX2* and *OSX* and late-stage markers *OCN*, *ONN*, *OPN*, and *BSP*, were evaluated by quantitative real-time PCR (Figure 19). The mRNA levels of all the genes assessed were significantly higher ($p < 0.001$) in the cells cultured on PCL-RSV_{10} and PLA-RSV_{10} membranes than in those seeded on the control membranes (PCL and PLA). As expected, the presence of resveratrol at 15 μM resulted in a consistently up-regulation of all selected genes in STRO-1^+ cells compared with cells cultured onto PCL and PLA membranes. These results are consistent with the previous reports that resveratrol promotes osteoblast differentiation and proliferation [3] [13, 148].

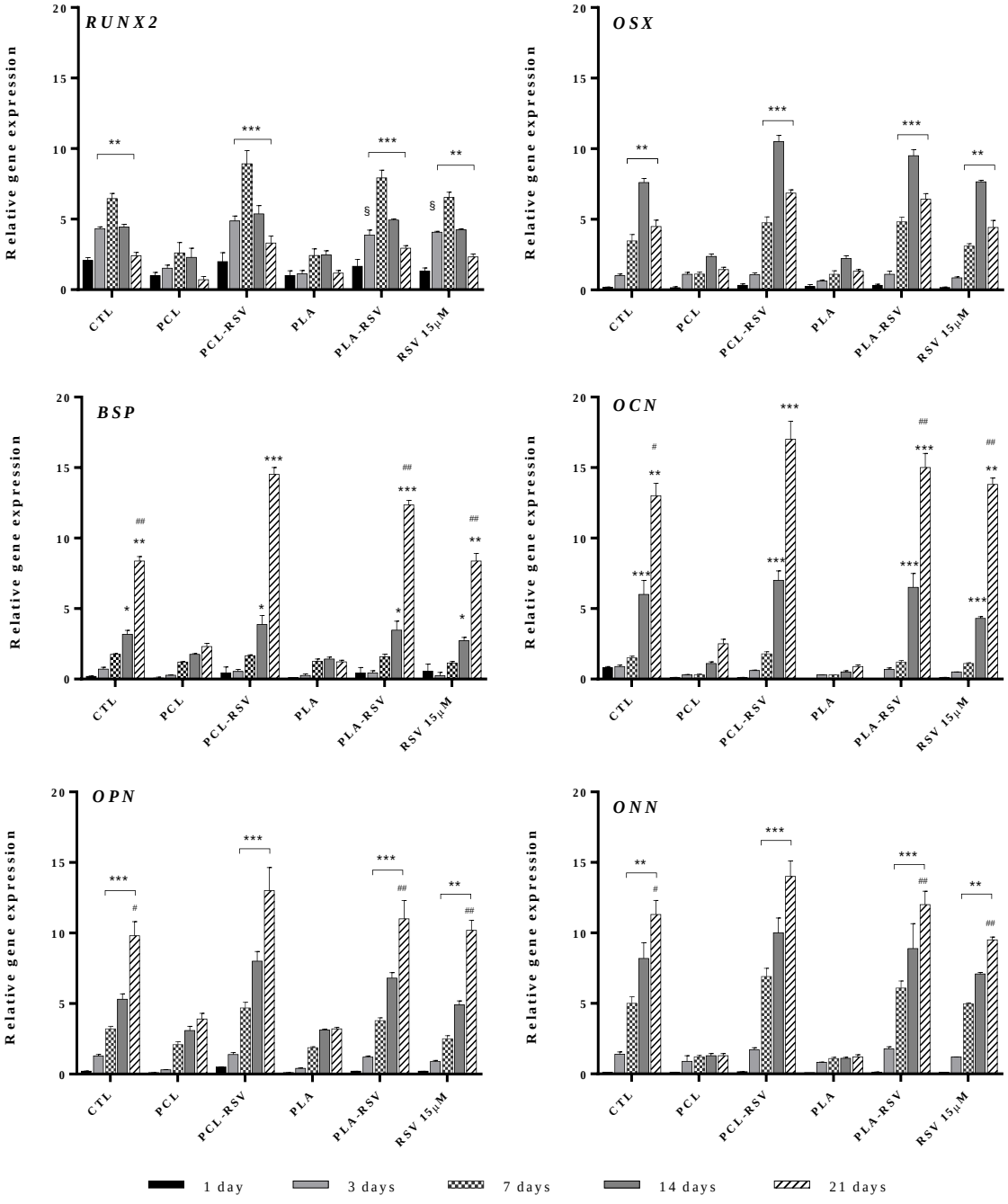

Figure 19. Quantitative RT-PCR analysis of *RUNX2*, *OSX*, *BSP*, *OCN*, *OPN*, and *ONN* in the STRO1^{+} cells cultured on membranes for 1, 3, 7, 14, and 21 days. The target gene expression was normalized to the housekeeping genes *HPRT1* and *GAPDH*. Relative differences in the PCR results were calculated using the comparative CT ($2^{-\Delta\Delta Ct}$) method. The bars represent the means ± standard deviation (n = 3). ***$p < 0.001$ *versus* PCL and PLA; **$p<0.01$ *versus* PCL and PLA; *$p<0.05$ *versus* PCL and PLA; ##$p < 0.01$ *versus* PCL-RSV$_{10}$; #$p < 0.05$ *versus* PCL-RSV$_{10}$.

6.5 Effect of resveratrol on osteoclast differentiation

Bone remodeling depends on a delicate balance between the bone extracellular matrix synthesis by osteoblasts and extracellular matrix resorption by osteoclasts [186]. Osteoclasts are specialized cells derived from the monocyte/macrophage haematopoietic lineage that develop and adhere to bone matrix, then secrete acid and lytic enzymes that degrade it in a specialized, extracellular compartment [187]. Osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) are newly discovered molecules that play a key role in the communications between osteoclasts and osteoblasts. RANKL is essential for osteoclast differentiation via its receptor RANK located on the osteoclast membrane, while OPG is a soluble decoy receptor that inhibits osteoclast differentiation through its binding to RANKL [4]. In physiological milieu, the binding of RANKL to its receptor RANK on the surface of osteoclast precursors leads to the activation of TNF receptor-associated factor 6 (TRAF6), which subsequently stimulates the transcription of osteoclast associated genes (such as cathepsin K) and leads to the formation of multinucleated tartrate-resistant acid phosphatase (TRACP) positive giant cells [186] [188].

He et al. reported the role of resveratrol in the inhibition of osteoclast generation and function suppressing RANKL-induced reactive oxygen species (ROS) generation in a concentration dependent manner [4]. Shakibaei et al. demonstrated the RANKL-induced acetylation and nuclear translocation of NF-κB in a time and concentration dependent by resveratrol. In addition, activation of Sirt-1 (a histone deacetylase) by resveratrol induced Sirt-1-p300 association in bone-derived and pre-osteoblastic cells, leading to deacetylation of RANKL-induced NF-κB, inhibition of NF-κB transcriptional activation and osteoclastogenesis [189].

To evaluate the effect of resveratrol concentration on RANKL-induced osteoclast formation, cells were incubated in presence of different concentration of resveratrol (0, 0.5, 1, 2.5, and 5 μM) after stimulation with RANKL for the indicated time. As shown in figure 20, the number

of TRAP-positive cells were reduced in a concentration- and time-dependent manner. In particular, RANKL-stimulated cultures displayed markedly lower TRAP activity after 14 day of culture in presence of 1 and 2.5 μM resveratrol compared to control cells. Complete inhibition of osteoclastogenesis was observed at 5 μM resveratrol concentration. Nonetheless, the viability of cells under these conditions was not affected at the concentrations used in this study (data not shown).

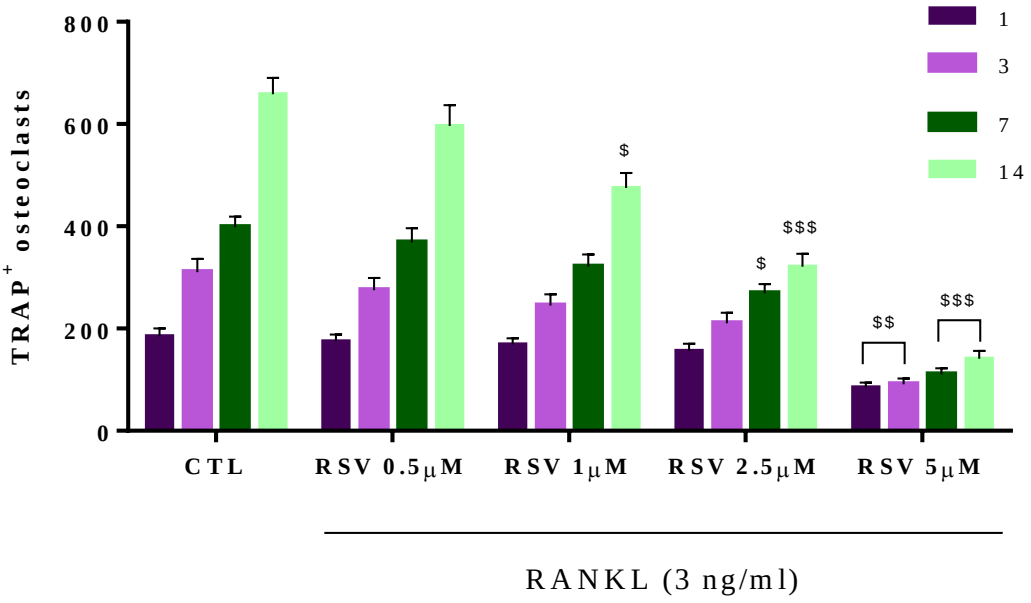

Figure 20. RSV inhibited RANKL-induced osteoclast differentiation. Cells were incubated in presence of different concentration of resveratrol (0, 0.5, 1, 2.5, and 5 μM) for 14 days with or without the addition of RANKL (3ng/ml) and then immunostained for TRAP expression. Cell cultured in presence of medium alone were used as control. TRAP-positive multinucleated cells having three or more nuclei were considered as osteoclasts; and the number of osteoclasts was counted after taking randomly fields in different areas of each well. At least 4 wells were used for each tested reagent and the values are expressed as mean ± SD. ($$$$p$ < 0.001, $$$p$ < 0.01, $$p$ < 0.05 *versus* CTL)

Further, the expression of cathepsin K, a specific osteoclast marker, was measured in cells cultured onto PCL, PCL-RSV_{10}, PLA, and PLA-RSV_{10} membranes for 14 days. As shown in figure 21, the expression of cathepsin K was downregulated at mRNA level in cells cultured

onto RSV-loaded membranes. This result indicates that RSV released from PCL and PLA electrospun membranes was able to directly inhibit RANKL-induced osteoclastogenesis.

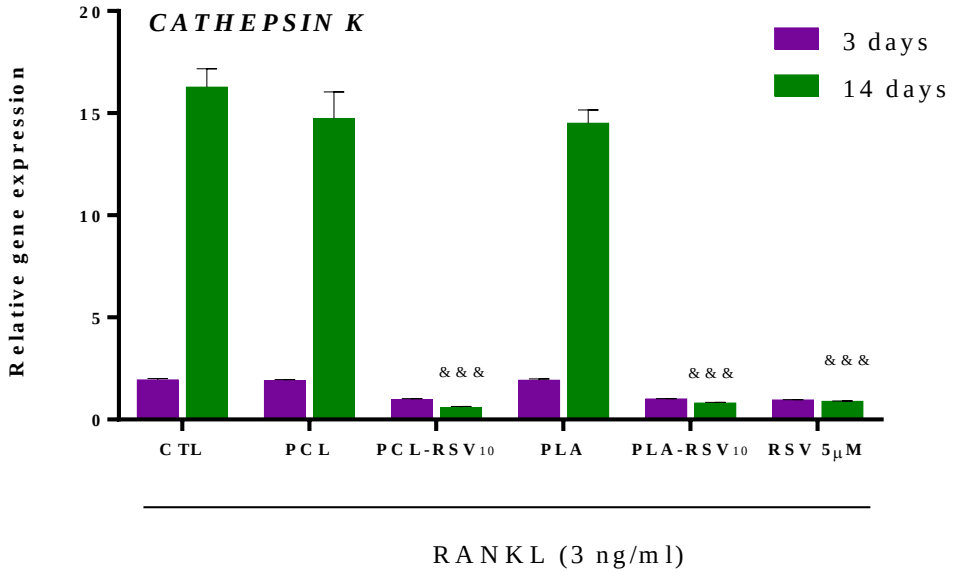

Figure 21. Quantitative RT-PCR analysis of *CATHEPSIN K* in cells cultured on membranes for 3, and 14 days. The target gene expression was normalized to the housekeeping genes *HPRT1* and *GAPDH*. Relative differences in the PCR results were calculated using the comparative CT ($2^{-\Delta\Delta Ct}$) method. The bars represent the means ± standard deviation (n = 3). &&&$p < 0.001$ *versus* CTL, PCL and PLA

The reported results demonstrated that the resveratrol-incorporated membranes were able both to control STRO-1^{+} cells differentiation toward osteoblast lineage and to inhibit RANKL-induced osteoclastogenesis.

Conclusions

7. Conclusions

In conclusion, our results provide evidence that resveratrol dispersed into electrospun fibers generates bioactive materials able to release resveratrol in a sustained manner and to enhance the osteogenic differentiation of DPSCs useful to improve GBR surgical procedure. Differentiation of DPSCs into osteoblasts as well as the inhibition of osteoclastogenesis are important aspects to promote the amount and quality of newly synthesized bone. Thus, our molecule releasing scaffolds have a promising potential in preserving the ridge volume within the bony envelope existing at the time of extraction modulating the resorption and remodeling of the alveolar ridge after tooth removal.

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8. References

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