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Effect of resveratrol release kinetic from electrospun nanofibers on osteoblast and osteoclast differentiation

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ABSTRACT

Resveratrol (RSV) has been shown to exhibit many biological properties that can influence bone osteogenesis. However, RSV oral clinical treatment is limited due to its poor pharmacokinetics, low water solubility, and rapid metabolism. Therefore, it is necessary to develop a valid delivery system to release RSV directly into the target site. Electrospun drug-eluting fibers have gained great attention in the regenerative dentistry due to the ease of fabrication, the high surface to volume ratio and the drug-loading efficiency. The post-extraction preservation of the alveolar socket requires to operate on the bone remodeling processes both by stimulation of bone formation by osteoblasts and inhibition of osteoclast-mediated bone resorption. In this work, uniform defect-free fibers of poly(ε-caprolactone) PCL and poly(lactic) acid (PLA) loading resveratrol were synthetized and characterized. *In vitro* assay demonstrated that the two membranes were able to release RSV in a tunable and sustained manner with different kinetic: PCL-RSV membrane showed an initial burst followed by a slow release, while PLA-RSV presented a much slower and continuous release over the time. Although both RSV-loaded materials showed similar *in vitro* osteoinductive capacity on human dental pulp stem cells, the differences on RSV release kinetic affected RANKL-induced osteoclastogenesis. Indeed, only the lower resveratrol-releasing membrane (PLA-RSV) was able both to induce osteoblast and to inhibit osteoclast differentiation, suggesting that this bioactive membrane could be used to preserve post-extraction alveolar ridge volume acting simultaneously on two fronts: first counteract bone resorption, second allows new bone formation.

1. Introduction

Resveratrol (RSV) is a naturally polyphenolic compound present in red wine and in numerous plants. Several biologic effects of resveratrol have been reported, including cardiovascular protection [1], and anticancer [2], anti-inflammatory [3], antioxidant [4], antiaging [3], and bone-protective activities [5]. In addition, RSV could enhance osteogenesis and inhibit adipogenesis in mesenchymal cell lines or bone marrow–derived mesenchymal stem cells (MSCs) [6,7]. Indeed, resveratrol, in a dose-dependent manner, is able both to stimulate osteoblast differentiation, probably by the activation of MAPK signaling pathways [8], and to inhibit bone resorption by the inhibition of RANKL-induced osteoclast differentiation. Interestingly, while RSV amount able to stimulate osteoblast growth and differentiation ranges between 10 and 100 μM, the osteoclast differentiation is inhibited by RSV at a concentration ranging between 0.3 and 5 μM [9,10].

For all these biological properties, RSV has been proposed for the treatment of the alveolar socket reduction and remodeling that can affect correct dental implant placement after tooth removal [11,12]. Dental clinicians have attempted to preserve the alveolar ridge after tooth extraction by using growth factors, such as recombinant human platelet derived growth factor (rhPDGF) [13], and recombinant human bone morphogenetic protein 2 (rhBMP-2) [14]. Although rhBMP-2 has been considered the most favorable osteogenic agent, according to the results of randomized clinical trial studies [15], translating clinical outcome into clinical practice has been prevented by rhBMP-2 side effects, such as swelling, seroma, and an increased cancer risk, as well as by the cost of the therapy [16].

However, despite the demonstrated ability of RSV in modulating BMPs and osteopontin gene expression in an animal model of bone repair [17], the clinical implications of these results remain controversial. In recent clinical trials, orally administrated RSV failed to

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show any significant effect on a panel of biomarkers of bone turnover and calcium metabolism [18,19]. The poor pharmacokinetics of RSV as well as its low water solubility, its rapid metabolism, and its chemical instability could explain, at least in part, the difficulty to identify RSV potential physiological effects *in vivo* after RSV oral administration.

A valid alternative to oral route could be a local controlled delivery system, such as electrospinning biodegradable membranes, able to release RSV directly into the alveolar bone defect.

Electrospun scaffolds have received growing attention in several fields of medicine [20] such as wound healing [21], drug delivery [22–25], and tissue engineering [26]. Electrospinning process allows to produce from a wide range of polymers continuous polymer nanofibers, resulting in a scaffold with high porosity and interconnected pores [27,28]. The surface topography, fiber morphology and orientation are largely dictated by solution properties (i.e. polymer molecular weight and concentration, conductivity of the solution, etc.) and operating conditions (i.e. applied voltage, solution flow rate, tip-collector distance, temperature, humidity, etc.). However, to verify whether the nanofiber delivery platform is a good candidate for clinical application, it is fundamental both to analyze in detail the kinetics of release of the bioactive molecule loaded into the polymer and to study the biological effects of its continuous delivery on *in vitro* cell targets. This is even more evident in the case of RSV whose biological activity is dose dependent and target-cell dependent.

In this study, a RSV release system was created using poly(ε-caprolactone) PCL or poly(lactic) acid (PLA) as the basic materials for nanofibers production. Physico-chemical characterizations were carried out to examine the drug release ability and the cytocompatibility of the scaffold. *In vitro* models were established: (1) to detect the kinetics of RSV delivery from nanofibers; (2) to evaluate long term ability of RSV released into the culture medium to promote stem cells differentiation toward an osteoblast phenotype; and (3) to investigate the inhibition of osteoclast generation by RSV.

Both materials were able to promote human dental pulp stem cells (DPSCs) differentiation into osteoblast-like phenotype, increasing gene expression of the osteogenic markers and inducing a marked calcium deposition after 28 days of incubation. However, only the lower resveratrol-releasing membrane (PLA-RSV) was able to inhibit RANKL-induced osteoclast differentiation by reducing TRAP activity and catephsin K gene expression. The PLA-RSV capability of modulating both osteoblast (positively) and osteoclast (negatively) differentiation is of particular interest in dentistry applications, such as post-extraction alveolar ridge preservation, limiting the physiological reduction and remodeling process that could affect correct implant placement. Our study may provide an experimental basis for new treatments for repair of alveolar bone defect.

2. Materials and methods

2.1. Preparation of resveratrol-loaded membranes using electrospinning

Poly(ε-caprolactone) (PCL, molecular weight of 80,000 Da) and Poly (L-lactide-co-D,L-lactide) (PLA, L-lactide:D,L-lactide = 70:30, inherent viscosity: 3.3–4.2 dl/g solutions) electrospinning membranes were prepared as reported in Valarezo et al. [29] by dissolving neat polymers in hot acetone (35–40 °C) at 14% and 2.5% (w/w), respectively. Resveratrol was added to PCL or PLA at the drug to polymer ratio of 0.1:9.9 (w/w) and mixed by Ultra Turrax T25 (Janke & Kunkel, IKA Labortechnik, Germany) for 5 min at 15,000 rpm to obtain a homogenous solution. Electrospinning conditions were reported in Table 1 and optimized to produce nanofibrous mats without bead formation. For convenience, the membranes obtained from PCL or PLA with RSV were hereafter defined as PCL-RSV or PLA-RSV.

Table 1
Processing parameters of fibers fabricated by electrospinning (flow rate: 4 mL/h).

Sample	Polymers concentration (wt%)	Nanofiber collect distance (cm)	Spinning voltage (kV)
PLA	2.5	30	30
PLA-RSV	2.5	30	30
PCL	14.0	30	30
PCL-RSV	14.0	30	30

2.2. Membranes characterization

The morphology and the diameter of the electrospun nanofibers were analyzed by a scanning electron microscope (SEM; JEOL JSM-T300). 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 ScanPro 5. For each membrane, 75 diameter measurements were considered, taking their dimension respect to the reference bar of SEM image.

X-ray diffraction (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 3 s of counting time.

Differential Scanning Calorimetry (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.

2.3. In vitro RSV release measurement

For drug release determination, samples were circular punches in pieces of 30 mm, vacuum-dried for 24 h and individually weighed. All samples were placed into individual vials and covered with aluminum foil to prevent drug degradation caused by light. Release kinetic was performed in artificial saliva medium (SAGF, 10 mL pH 6.8, Sigma-Aldrich) at 37 °C. At predetermined time intervals (every hour for 8 h, then every day for 10 days, and then every week over a 35-day period), supernatants were collected and stored at 4 °C until analysis. The resveratrol concentration was measured using HPLC-UV procedure according to Omar et al. with some modifications [30]. The analysis was carried out on Agilent 1260 Infinity Quaternary LC (Agilent Technologies) 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) protected by a guard column (Security Guard Cartridge C18, 4 × 2.0 mm inner diameter, Phenomenex) 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 re-equilibrated for 5 min with the initial solvent. The detection wavelength was set at 290 nm and RSV quantitation was 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. Prior to injection, the standards and samples were filtered through a 0.22 µm pore-size filter (Millipore). System control and data acquisition were performed using the ChemStation software (Agilent Technologies).