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Original article

Antiangiogenic activity of PLGA-Lupeol implants for potential intravitreal applications

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ABSTRACT

Uncontrolled angiogenesis is directly associated with ocular diseases such as macular degeneration and diabetic retinopathy. Implantable polymeric drug delivery systems have been proposed for intravitreal applications and in the present work, we evaluated the antiangiogenic potential of PLGA ocular implants loaded with the triterpene lupeol using *in vitro* and *in vivo* models. The drug/polymer physiochemical properties of the lupeol-loaded PLGA were validated as functionally similar using differential scanning calorimetry, Fourier transform infrared spectroscopy, and scanning electron microscopy. Interestingly, in an *in vitro* culture system, lupeol (100 μg/mL and 250 μg/mL) was capable to inhibited the proliferation as well as the migration of Human Umbilical Vein Endothelial Cells (HUVEC), without interfering in cell viability, promoting a significant reduction in the percentage of vessels (39.41% and 44.12%, respectively), compared with the control group. *In vivo* test, by using the chorioallantoic membrane (CAM) model, lupeol-loaded PLGA ocular implants showed antiangiogenic activity comparable to the FDA-approved anti-VEGF antibody Bevacizumab. Overall, our results suggest lupeol-loaded PLGA ocular implants were able to inhibit the angiogenic process by impairing both proliferation and migration of endothelial cells.
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1. Introduction

Angiogenesis is a natural process that occurs in the human body during fetal development or as a response to tissue damage, as part of wound healing process and renewing the blood flux in damage areas [1,2]. When the human body loses its capacity to maintain adequately the equilibrium of angiogenic mediators, some diseases can develop, including arthritis, cancer, endometriosis, psoriasis, macular degeneration related to age and proliferative diabetic retinopathy [3,4]. Macular degeneration related to age is the main cause of blindness in persons older than 60 years in industrialized countries [5]. The prevalence of blindness can vary between 10 and 15% among affected persons and estimates have shown a significantly rates increase until 2030 [6]. Currently, available treatments are limited to palliatives such as photodynamic therapy, intravitreal injections with corticoids, antiangiogenic compounds applied directly into eye and laser photocoagulation [7–10]. The proliferative diabetic retinopathy is characterized by new important vascularization in retina, following to intravitreal interface, with possible loses of normal visual characteristics mainly due to traditional retinal detachment. In this sense, the inhibition of angiogenesis can be considered an important strategy to treat these intraocular diseases and have been explored in many published works [11–14].

Lupeol is a natural pentaciclic triterpene with a lupane scaffold that can be found in diverse vegetables, including white cabbage, red pepper, cucumber, tomatoes, carrots, peas, and soy. Moreover, administration of lupeol does not result in systemic toxicity in animal models in doses ranging from 30 to 2000 mg kg^{-1} [15].

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Triterpene can be isolated from medicinal plants, *i.e. Celastraceae* family plants, displaying clinically relevant biological properties related to inflammation, arthritis, cardiovascular disorders, cancer and wound healing processes [16–18]. You et al. [19] evaluated the antiangiogenic activity of lupeol on a tube-like formation assay using HUVEC cells (*Human umbilical vein endothelial cells*). The results revealed lupeol capacity to inhibit 80% of angiogenic processes at a non-toxic dose of 50 $\mu g\,mL^{-1}$, whereas lower doses showed a significant reduction in antiangiogenic activity reaching only 40% of initial values. Other studies using an endothelial cell model have indicated that triterpenes can modulate growth factors such VEGF (Vascular endothelial growth factor) and are capable of inducing cellular differentiation, seeking to inhibit the vascular tissue growth, displaying an important antiangiogenic effect [20–23].

Due to anatomical and physiologic characteristics of the eye, administration of ophthalmic medicines is difficult and many studies showed that only approximately 5% of the administrated dose are absorbed by intraocular tissues, making the treatment unfeasible for diseases located in posterior segment of the eye. Other available treatments require the use of high drug doses or are too invasive as intravitreal injections, exhibiting great risks and potentially serious side effects to the patient [24–26]. Seeking to overcome this negative scenario, research has been dedicated to developing new drug delivery systems, such as polymeric implants with the overall goal to be more selective and achieve favorable bioavailability profiles through sustained releasing of the therapeutic cargo [27,28]. Such systems offer many advantages, including favorable patient compliance, biocompatibility, predictable biodegradation kinetic and mechanical resistance in various intravitreal applications [29–31]. In order to mitigate the cumulative risks associated with repeated intravitreal injections, some implantable polymeric systems have been approved by FDA and currently available, for example: Ozurdex® (Dexamethasone Intravitreal Implant); Iluvien® (Fluocinolone acetonide intravitreal implant) and Triesence® (Triamcinolone acetonide). In these systems, any cytotoxicity was observed as well as significant antiangiogenic activity were obtained, displaying the applicability of these systems in intravitreal applications. However, at present, no steroid has achieved US FDA approval for the treatment of pathologies associated to angiogenesis (except for ranibizumab, a monoclonal antibody).

In this work, we aimed to evaluate the anti-angiogenesis activity of PLGA ocular implants containing the lupeol, a non-steroid compound, in both *in vitro* and *in vivo* models.

2. Experimental

2.1. Chemical and reagents

Poly (D,L-lactide-co-glycolide) in ratio of 75:25 [PLGA (75:25)] was purchased from Boehringer Ingelheim (Germany). All the solvents and reagents used in buffer solutions, in the preparation of the implants, and mobile phase were HPLC or analytical grade. Water was distilled, deionized and filtered through a 0.22 μm filter (Millipore, USA).

2.2. Methods

2.2.1. Lupeol extraction

Dried and pulverized stem of *Maytenus salicifolia* (2525.9 g) were subjected to exhaustive maceration in *n*-hexane at room temperature, yielding 14.7 g of hexane extract. It was subjected to silica gel column chromatography (CC) eluted with *n*-hexane, $CHCl_3$, AcOEt, and MeOH, pure or in mixtures of enhanced polarity, yielding 52 fractions gathered in 14 groups. Group 8 (Fraction 31; 137 mg) was chromatographed on silica gel CC eluted with *n*-hexene/$CHCl_3$ (4:1) furnishing 25 fractions of 5 mL each. Fractions 5–12 providing a white solid (93.0 mg) which was identified as lupeol. The structural elucidation of this compound was based on its IR, ^1H and ^{13}C NMR spectral data.

2.2.2. Lupeol characterization

Lupeol was characterized by melting point, FTIR and NMR spectra. Melting point was uncorrected and measured using Thermo system FP800 Metler apparatus. FTIR spectrum were recorded on a Perkin-Elmer, FTIR Spectrum-1000 and NMR experiments were carried out on Bruker DRX400 Avance spectrometer, using TMS as an internal standard and $CDCl^3$ as solvent.

2.2.3. PLGA-lupeol implant preparation

Lupeol-loaded PLGA ocular implants were prepared according to method developed by Fialho and da Silva [32]. Briefly, 100 mg of PLGA 75:25 and 30 mg of lupeol were dissolved in 10 mL of acetone at room temperature. Then, the solution was placed in a freezer under −80 °C. Afterwards, the frozen solution was lyophilized for 30 h and the obtained material were then molded to rods using Teflon® sheets heated on a hot plate from 50 to 60 °C in order to form lupeol-loaded PLGA ocular implants with diameter of 0.45 mm and length of 6 mm, containing 30% lupeol w/w.

2.2.4. Physicochemical characterization

2.2.4.1. Differential scanning calorimetry (DSC). Differential scanning calorimetry analysis (DSC) DSC-50, Shimatzu DSC apparatus was used. The samples constituted by lupeol; blank PLGA 75:25 and lupeol-loaded PLGA ocular implants were heated in semi-hermetic aluminum pans, and the first scan was measured at a heating rate of 10 °C min^{-1} from room temperature to 180 °C. Subsequently, the samples were cooled to −100 °C and heated to 400 °C (second run) under nitrogen atmosphere.

2.2.4.2. Fourier transform infrared spectroscopy (FTIR). Fourier transform infrared spectroscopy (FTIR) Infrared spectra were collected in a Fourier transform infrared spectrophotometer (FTIR) (model Spectrum 1000; Perkin Elmer). Measurements were carried out using the attenuated total reflectance (ATR) technique. Each spectrum was a result of 32 scans with a resolution of 4 cm^{-1}, between ranging 4000–650 cm^{-1}. Samples were constituted by lupeol; blank PLGA 75:25 and lupeol-loaded PLGA ocular implants.

2.2.5. Morphological characterization

The structure of lupeol-loaded PLGA ocular implants was observed by scanning electron microscope TESCAN VEGA 3 LMU (Czech Republic). Samples characterized by 4 mm of length and 0.5 mm of diameter were gold-coated and imaging were conducted in voltage of 10 kV.

2.2.6. In vitro *antiangiogenic studies*

2.2.6.1. HUVEC cells culture and cytotoxicity evaluation. Human Umbilical Vein Endothelial Cells (HUVEC; ATCC – CRL-2873) were cultured in endothelial cell supplemented media (Endothelial Growth Medium, EGM-2; Lonza). Cells in the sixth or seventh passage were used in the experiments after incubation in deprivation medium (Endothelial Basal Medium, EBM-2; Lonza) containing 0.1% fetal bovine serum (FBS) overnight at 37 °C 5% CO_2, for cell cycle synchronization.

Lupeol was initially diluted in phosphate-buffered saline (PBS) containing 10% dimethyl sulfoxide (DMSO), and 10 mg mL^{-1} stock

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