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Original article

Development of thalidomide-loaded biodegradable devices and evaluation of the effect on inhibition of inflammation and angiogenesis after subcutaneous application

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ABSTRACT

Purpose: To develop thalidomide-loaded poly-lactide-co-glycolide implants and evaluate its *in vivo* release and biological activity against inflammation and angiogenesis after subcutaneous administration.
Methods: Implants were prepared by the hot molding technique and characterized using stereomicroscopy, thermal analysis and X-ray diffraction. Swiss mice, divided in groups 1–3, received a subcutaneous implant containing 25% (w/w), 50% (w/w) or 75% (w/w) of thalidomide, respectively ($n = 6$). The drug levels were determined during a 28-day study period. The toxicity associated with the implants was evaluated by light microscopy. The potential of the developed implant in the inhibition of inflammation and angiogenesis was evaluated *in vivo* using the sponge model.
Results: Thalidomide implant was developed and its characterization proved the stability of the drug and the polymer during preparation. Release profiles *in vivo* demonstrated an extended release of thalidomide from the implants during the 28 days. Histological evaluation did not show any sign of intense local inflammatory response to the presence of the implants in the subcutaneous pouch. The thalidomide implant reduced the number of vessels and N-acetyl-b-glucosaminidase (NAG) *in vivo*.
Conclusion: The biodegradable implants delivered safe doses of thalidomide that were also effective to induce angiogenesis and inflammation regression.

1. Introduction

Thalidomide (THD) was initially indicated as a sedative drug and the use by pregnant women led to severe infant limb defects probably because of its anti-angiogenic effect [1]. Besides, thalidomide presents important pharmacological properties, that include anti-inflammatory, immunomodulatory and antiangiogenic effects that shows remarkable value in the control or treatment of several diseases, even cancer [2,3].

Regarding its pharmacokinetics, thalidomide has physical–chemical properties that may be troublesome for bioavailability after oral administration. It is practically insoluble in water and presents a rapid and spontaneous hydrolysis that leads to an erratic and incomplete absorption after oral administration and even injectable formulations are limited face to thalidomide physicochemical properties [4–6].

Furthermore, thalidomide has severe adverse effects that limit its use, which includes peripheral neuropathy, constipation, sedation, somnolence, rash and deep vein thrombosis, resulting sometimes in treatment withdrawal [7].

A drug delivery system, in the form of biodegradable implants, may be an effective dosage form for the delivery of thalidomide since they may promote: (1) controlled drug release, that allows maintenance of effective therapeutic levels for a prolonged period of time without frequent doses dependent of patient adherence; (2) drug release directly to the site of action, avoiding adverse effects caused by systemic distribution;

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(3) drug protection from inactivation before reaching the site of action; (4) ease of application [8,9]. The biodegradable polymers are recognized as promising materials for the development of drug delivery systems since they do not require subsequent removal by surgery after the complete release of the drug [10,11].

Therefore, this study aimed to develop and characterize thalidomide-loaded poly-lactide-co-glycolide (PLGA) implants, to evaluate the *in vivo* drug release and also the effectiveness of the device to reduce angiogenesis and inflammation.

2. Methods

Thalidomide (THD, racemic form, purity 99.49%, α polymorph) was purchased from Microbiológica Química e Farmacêutica LTDA (Rio de Janeiro, Brazil). Poly-lactide-co-glycolide copolymer (PLGA 50/50, PURASORB® PDLG 5004, inherent viscosity midpoint of 0.4 dL/g) was a gift from Purac Biomaterials (São Paulo, Brazil). Acetone and acetonitrile HPLC grade were obtained from Merck Brasil (São Paulo, Brazil). Ultrapure water was produce by a Milli-Q System, Millipore (Massachusets, USA). Other chemicals were of analytical grade.

2.1. Preparation of thalidomide-loaded PLGA implants

The implants were prepared according to the technique previously described by Fialho and Silva-Cunha [12]. Thalidomide at 25% or 50% or 75% (w/w) and PLGA 50:50 were dissolved in a mixture of distilled water/solvent and lyophilized (−40 °C, Pirani501 78/L, Edwards Ltda., Brazil). The lyophilized powder was molded into rods using a hot plate. The implant obtained was 1 mm in diameter and 4 mm in length.

2.2. Characterization of thalidomide-loaded PLGA implants

2.2.1. Morphology

The morphology of the developed devices was evaluated without any special preparation by a stereomicroscopy (SZ61TR, Olympus, Brazil) at a magnification of 25×. Leica DM 4000 B software (Germany) was used for implant measurement.

The implants retrieved from the *in vivo* study were also visualized by stereomicroscopy for the evaluation of any changes in its structure during the 28-days period of the study.

2.2.2. Differential scanning calorimetry (DSC)

DSC analysis was accomplished with a Mettler Toledo differential scanning calorimeter (DSC822e, Mettler-Toledo International Inc., Brazil). Samples of about 2 mg (THD, PLGA and implants containing 25%, 50% or 75% (w/w) of THD) were accurately weighed in closed and pierced aluminum pans (40 μL). The curves were obtained in the temperature range of 25–400 °C, using 10 °C/min of heating rate and ultrapure nitrogen at 50 mL/min of purge gas.

2.2.3. X-ray diffraction

Implants patterns were collected on a Shimadzu diffractometer with a Cu Kα radiation at room temperature. Each sample (implants containing 25%, 50% or 75% (w/w) of THD and PLGA) was measured from 4° to 70° in 2θ using a graphite monocromator under 30 rpm of spinning to prevent any preferred orientation with step size of 0.02° 2θ.

2.3. In vivo *release study*

Female Swiss mice weighing approximately 30 g, 6–8 weeks old, were maintained in cages under a quiet and climatically controlled environment with free access to standard mice chow and water and light/dark cycle of 12/12 h. The animals were monitored for any signs of infection at the intervention site, discomfort or distress, or even weight loss; any mice presenting such signs were immediately sacrificed. The work has been carried out in accordance with EU Directive 2010/63/EU for animal experiments. The study was approved by the Ethics Committee in Animal Experimentation of Ezequiel Dias Foundation (Protocol n° 027/2011, Belo Horizonte, Brazil).

Animals were divided in three groups with six individuals each. Groups 1, 2 and 3 received implants with 25%, 50% and 75% (w/w) of THD, respectively. Before implantation, the mice were anesthetized with a mixture of xylazine (10 mg kg^{-1}) and ketamine hydrochloride (100 mg kg^{-1}) by the intraperitoneal route. The dorsal hair of the animals was shaved and the skin wiped with 70% (v/v) ethanol. Then, the implants were applied through a 1 cm incision made in the animal dorse.

After 3, 7, 14 and 28 days, the animals were sacrificed with lethal dose of pentobarbital. The implants were removed and their surface morphology was visualized. Additionally, at those times, the concentration of THD released *in vivo* was calculated indirectly from the retrieved implants by high performance liquid chromatography (LabChrom Elite UV/VIS, Merck Hitachi, Malta, NY; L-2130 pump, L-2200 autoinjector, L-2300 oven, L-2400 UV detector) equipped with an octadecylsilane column (C18, 150 mm × 3.9 mm, 5 μm particle size; XTERRA® MS C18, Waters, Milford, MA) thermostated at 25 °C and a UV detector set at 237 nm. The mobile phase was composed of acetonitrile: phosphoric acid 0.1% (20:80) at a flow rate of 1.2 mL/min. In these conditions, the retention time of THD was about 5.5 min. The area of the THD peak was reported to a calibration curve for the determination of THD concentration.

2.4. Histopathologic evaluation

At the end of the 28 days of the *in vivo* release study, the subcutaneous tissue of the sacrificed animals (1 cm at each direction from the implant), including the implants without drug or containing 75% (w/w) of THD, was carefully removed and fixed in phosphate buffered 10% neutral formalin. The tissue was processed by the routine technique of paraffin embedding. Histological sections (4 μm) underwent hematoxylin-eosin (HE) staining and were observed using an optical microscope (Leica Microsystems, Germany) at 50× or 100× magnification.

2.5. Inhibition of inflammation and angiogenesis in a murine sponge model

2.5.1. Implantation of the sponge discs

Other female Swiss mice weighing approximately 30 g, 6–8 weeks old were used in this study. They were maintained at the same conditions as described in item 2.3 *In vivo* release study. This study also followed the EU Directive 2010/63/EU for animal experiments and was approved by the Ethics Committee in Animal Experimentation of Ezequiel Dias Foundation (Protocol n° 027/2011, Belo Horizonte, Brazil). The animals were divided in two groups of eight animals each; one group received the implants containing THD (treated group) and the other received the implants without drug (control group).

The non-biocompatible sponge discs used as the matrix for fibrovascular tissue growth were 5.0 mm in thickness, 8.0 mm in diameter and approximately 4.5 mg in weight (Vitafoam Ltd, Manchester, UK). They were soaked overnight in 70% (v/v) ethanol and sterilized by boiling in distilled water for 15 min before implantation. The implants containing 75% (w/w) of THD were incorporated into these sterilized sponge discs.