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Original article

Retinoic acid-loaded alginate microspheres as a slow release drug delivery carrier for intravitreal treatment

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ABSTRACT

Pharmacotherapy were the mainstream methods for the treatment of proliferative vitreoretinopathy (PVR), one of the leading causes of blindness. Due to the negligible side-effects, retinoic acid (RA) was considered to be a promising candidate drug. However, it was still a challenge to deliver drugs into vitreous with both long-term efficiency and biosafety. Sodium alginate was considered to be an excellent drug carrier owing to its controlled size, good biocompatibility and degradability. Based on a one-time synthetic strategy of preparing RA-sodium alginate microspheres, the study was aimed to establish a non-toxic and effective RA slow release platform. The prepared microspheres were smoothly round and relatively homogenous with an average diameter of 95.7 ± 9.6 μm. RA release rate from RA-MS was assessed *in vitro* by UV spectrometry and *in vivo* by high performance liquid chromatography, achieving a steady, long-term and effective level of RA. The biocompatibility of RA-MS was further confirmed through histology using HE staining and fundus microscope, finding no inflammation and other toxic response. Collectively, it could be concluded that RA-MS possessed a great potential in retinal PVR treatment.

1. Introduction

Proliferative vitreoretinopathy (PVR) is the leading cause of blindness and failure in retinal detachment surgery [1,2]. Pharmacotherapy was one of the most common treatment for PVR. Currently, There were abundant drugs available now, such as cyclosporine A [3], heparin [4] and retinoic acid(RA) [5]. Compared with other drugs, RA has gradually attracted a wide attention [6] because of its good biocompatibility and non-toxicity. In fact, RA naturally existed in the body as one of vitaminA derivatives and also the intermediate metabolites of photoreceptor [7]. Previously, much effort has been made on the drug delivery for PVR treatment. However, due to the existence of blood-ocular barrier, the therapeutic effects on intraocular diseases through holistic and local medicine injection at external part of eyeballs were usually limited. The intraocular, mainly intravitreal, drug delivery was thus considered to be a preferred method [8]. The loss of delivered drug from vitreous cavity was another issue limiting the efficacy of pharmacotherapy. In order to maintain the effective drug level, patients need to receive frequent injections, causing much pains and potential complications.

To overcome these drawbacks above, the intravitreal one-time drug delayed release system was proposed and extensively investigated. The dissolvability of RA in an intraocular filling (such as silicone oil) provided a sustained release strategy which may preserve drug level for a prolonged period. After dissolving in silicone oil, RA could still maintain its anti-proliferative effect without losing any stability [5]. However, this method was problematic that only the patients who need silicone oil treatment can be appliedto and long-term treatment with fluorinated silicone oil can damage the retina [9]. In addition, the property of silicone oil also suppressed the spread of drugs. Currently, a novel and substitutive slow-release carrier for RA was urgently needed. In recent years, lactic acid-glycolic acid copolymer (PLGA) was considered as a candidate carrier of RA for intraocular therapy due to its good biodegradation However, localized foreign body reactions can be detected using PLGA, limiting its further utility [10]. Therefore, it is still essential to develop biocompatible and effective drug delivery systems for intraocular administrations.

Sodium alginate, extracted from natural plant brown algae and composed of β-D-mannose and α-L-glucose, were capable of crosslinking (curing) after introducing Ca^{2+}. The size and morphology of crosslinked sodium alginate could be tuned via different synthetic protocols. Moreover, Ca^{2+} in alginate could diffuse out gradually, leading to disintegration and non-toxic degradation of alginate [11]. Because of these good properties, sodium alginate has been employed to protect implanted cells from immunological rejection, such as islet cells [12] and parathyroid cells [13]. Additionally, sodium alginate possessed

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tremendous potentials in drug delivery. It has been demonstrated that various drugs, such as antibiotics[14], cytokines [15], antitumor drugs [16] and even proteins [17], could be encapsulated into sodium alginate, improving the bioavailability of drugs. Recently, a research demonstrated that porcine retina pigment epithelium (RPE) cells could propagate with sodium alginate while maintain normal morphology, indicating its biocompatibility to retina [12]. Nevertheless, sodium alginate has not yet been applied to carry RA for intraocular administrations.

Here, we prepared RA-sodium alginate microspheres (RA-MS) under sterile condition for the first time. The release characteristics of RA-MS were systemically investigated *in vitro* and *in vivo*. Also, the biological toxicity of RA-MS was examined by pathology and electrophysiology.

2. Materials and methods

2.1. Chemicals

Alginate sodium and retinoic acid were purchased from Sigma-Aldrich. Cadmium chloride ($CaCl_2$), Dimethylformamide (DMF), Methanol, acetic acid, ethanol, acetone and other chemicals were purchased from Sinopharm Chemical Reagent co., Ltd, Chinawith analytical purity or higher.

2.2. Animals

Chinchilla rabbits (1.5–2.3 kg) were received from Laboratory Animal Center of Chinese PLA General Hospital as gifts. Randomly-picked nine animals were used in drug toxicity assay.

Pre-ophthalmologic examination: no conjunctival congestion, transparent cornea, normal depth of the anterior chamber, transparent lens and vitreous, brown eye fundus with uniform and clear distribution of fundus pigment, and no hyperemia and edema in eye fundus.

2.3. Synthesis of microspheres

Alginate microspheres containing RA (RA-MS) were produced as follows: a solution containing 0.05 M NaOH in 0.2 M HEPES was mixed with 7.5% $NaHCO_3$ (18% final volume) and RA under ice bathing. The RA-containing solution was then mixed with equivolume 1.5% alginate solution. Electrostatic droplet generator (provided by the University of Shanghai for Science and Technology, Shanghai, China) was set at flow rate of 10 mL/h and electrostatic potential of 7 kV. Simultaneously, the droplets underwent gelation with100 mM $CaCl_2$ gentle magnetic stirring. Then, another 15 min was required for solidification. Ultimately, the RA-MS was harvested after washing three times. The control MS were attained by the same procedure only without the addition of RA. Morphologies of microspheres were observed under light-microscope. A total amount of 250 microspheres were selected and measured via micrometer to obtain the average and distribution of diameters.

2.4. Drug loading assay

The weighted microspheres were dissolved in 1 mL DMF and shaken around 5 min. The mixture were then centrifuged under 4000 rpm for 5 min. The supernatant were applied in UV–vis spectrometer (Shimadzu, Japan). The optical densities (ODs) at 352 nm were recorded. Standard curve of RA were then established and the equation between ODs and concentration (C) of RA was showed below:

$$OD = 0.24C + 0.12 \ (R^2 = 0.99)$$

The total mass of RA dispersed in mL DMF was then calculated based on the obtained concentration. Finally the amount of drug-loading was expressed as ug/mg (RA/RA–MS).

2.5. Drug releasing assay in vitro

The drug releasing assay was performed according to a previous study [18]. Briefly, 30mgRA-MS was dispersed in 5 mL 1 × PBS (pH7.4, Thermal Fisher Scientific, USA) and stored at room temperature. The solvent was replaced for ten times per day at the set time. The OD of supernatants and RA-MS were analyzed at day 0, 3, 7, 14 and 28. Each sample was repeated three times.

2.6. Injection of microspheres into vitreous

The injection procedures followed a previous protocol [19]. After anesthesia with ketamine (50 mg/kg) and phenergan (25 mg/kg), both pupils of the rabbit eyes were dilated using 1% atropine and 1%compound tropicamide eye drops (tropicamide 0.3%, phemylephrine hydrochloride 0.3%, Zhongshan Ophthalmic Center, China).1% Tetracaine eye drops (Zhejiang Jiuxu Pharmaceutical Co., Ltd., China) was used for topical anesthesia followed by the puncture of anterior chamber. 0.3–0.5 mL of aqueous humor was aspirated to avoid the increase of intra-ocular tension. The eyeballs were immobilized with toothed forceps. Under surgical microscope, No. 4.5 needle (outlet: 0.46 mm, inlet: 0.25 mm) was inserted from pars plana which was 3.5 − 4.0 mm behind corneal limbus of superior temporal or superonasal. The needle was directed towards the mid-vitreous cavity. Subsequently, 15 mg washed RA-MS or MS in 0.4 mL PBS was injected. Finally, the conjunctival sac was painted with chlorotetracycline eye ointment. All animals underwent daily eye fundus and anterior chamber examination as well as antibiotic eye drop for a week after the procedure.

2.7. Electrophysiology

After injection for 1, 2, 3, 4 and 6 weeks, the rabbits received electroretinogram (ERG) and visual evoked potential (VEP) examination, respectively. The value of ERG-b and VEP were compared between experimental and normal eyes.

Flash electroretinogram (F-ERG) and flash visual evoked potential (F-VEP) were also conducted, as follows:

F-ERG: Typically, after anesthesia, pupils of rabbits were dilated using 1% compound tropicamide eye drops. After 30 min dark adaption, animal topical corneal was anesthetized with 1% Tetracaine eye drops. Corneal contact lens electrode was placed and attached to the center of corneal trough 0.5% methylcellulose. Reference electrode and grounding electrode were inserted in the middle of two eyes and the same side subcutaneous tissues of ear, respectively. The corneal plane was 5 cm far away from Gansfieldstimulator. Single flash stimulation was utilized. The stimulator generated white flash signal (30 Hz, 20 times).The amplitude and time to peak of wave-a, b as well as the wave shape of ERG were recorded and printed. Then, the amplitude and latent period of wave-b were also recorded. Each result was repeated at least 3 times.

F-VEP: The recording electrode was set at the middle of two ears, while reference electrode was placed at the center of forehead and grounding electrode was inserted in mandible. Similarly, after adapting in darkness, 2 Hz while optical signal was produced by flash stimulator for 60 times. Receiving electrode at occiput input amplified electric potential into computer. After 60 times superimposing, the amplitudes and time values were recorded and printed.

2.8. Gross anatomy

The gross anatomy was performed at day 3 and 7 post-injection using surgical microscopy, in order to observe the changing of vitreous and retina.