



## In vivo inhibition of hypertrophic scars by implantable ginsenoside-Rg3-loaded electrospun fibrous membranes



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### ABSTRACT

Clinically, hypertrophic scarring (HS) is a major concern for patients and has been a challenge for surgeons, as there is a lack of treatments that can intervene early in the formation of HS. This study reports on a Chinese drug, 20(R)-ginsenoside Rg3 (GS-Rg3), which can inhibit *in vivo* the early formation of HS and later HS hyperplasia by inducing the apoptosis of fibroblasts, inhibiting inflammation and down-regulating VEGF expression. Implantable biodegradable GS-Rg3-loaded poly(L-lactide) (PLA) fibrous membranes were successfully fabricated using co-electrospinning technology to control drug release and improve drug utilization. The *in vivo* releasing time of GS-Rg3 lasts for 3 months, and the drug concentration released in rabbits can be controlled by varying the drug content of the electrospun fibers. Histological observations of HE staining indicate that GS-Rg3/PLA significantly inhibits the HS formation, with obvious improvements in terms of dermis layer thickness, epidermis layer thickness and fibroblast proliferation. The results of immunohistochemistry staining and Masson's trichrome staining demonstrate that GS-Rg3/PLA electrospun fibrous membranes significantly inhibit HS formation, with decreased expression of collagen fibers and microvessels. VEGF protein levels are much lower in the group treated with GS-Rg3/PLA electrospun membranes compared with other groups. These results demonstrate that GS-Rg3 is a novel drug, capable of inhibiting the early formation of HS and later HS hyperplasia. GS-Rg3/PLA electrospun membrane is a very promising new treatment for early and long-term treatment of HS.

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### 1. Introduction

Hypertrophic scarring (HS) is a dermal fibroproliferative disorder that often occurs following surgical incision, deep trauma or severe empyrosis, and it has been a major concern for patients and a challenge for surgeons for centuries. Clinically, HS is raised, red and pruritic, and it frequently results in significant functional and cosmetic impairment, which together cause a decrease in the quality of life [1]. In the developed world, 4 million patients acquire scars resulting from burns each year, and the incidence is even greater in the developing world [2–4].

Vast numbers of studies have shown that abnormal patterns of tissue cellularity, exaggerated inflammation, overabundant extracellular matrix (ECM) production and accumulation, augmented neovascularization and reduced apoptosis are some of the major characteristic features of HS [5]. Treatments targeting these pathologic processes are very important in the prevention and inhibition of HS. Currently, there are many preventive and therapeutic options, such as silicone, pressure therapy, corticosteroids, laser therapy, cryotherapy, radiation and surgery. Some new methods have also been developed, such as interferon and 5-fluorouracil injection. Among these options, drug therapy is easy to commence and thus favorable to patients [6]. Corticosteroids and 5-fluorouracil are now the two most popular injectable HS treatments. 5-Fluorouracil causes a general suppression of the exaggerated inflammatory process and the proliferative process, whereas corticosteroids act by suppressing the inflammatory process only. However, injections of corticosteroids or 5-fluorouracil are not ideal, because they are associated with several unwanted disadvantages, including: (1) the induction of skin atrophy, depigmentation and telangiectasias;

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(2) the induction of an annoying burning sensation; and (3) requirement for repeat injections over several months [7].

To overcome the first disadvantage mentioned above, it is of great importance to search for novel drugs that do not cause unwelcome side effects. 20(R)-ginsenoside Rg3 (GS-Rg3) is one such Chinese drug, which has the well-known bioactive principles of *Panax ginseng*. It has been of great interest in studies of tumor therapeutics in recent years [8]. Some reports have indicated that it can suppress the production of some proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, and it may thus inhibit exaggerated inflammation during the early phase of HS formation [9,10]. Recently, GS-Rg3 was found to be capable of inducing the apoptosis of human fibroblasts [11,12]. In addition, studies have also shown that GS-Rg3 can inhibit xenograft growth and angiogenesis in tumors, primarily via the down-regulation of VEGF expression [13,14]. Therefore, the present authors speculate that GS-Rg3 might be a potential agent capable of preventing or reducing HS formation.

Sustained-release drug delivery systems have long been used in various fields to deliver drugs at a controlled rate over a period of time to the site of action [15]. To overcome the second and third disadvantages mentioned above, such a drug delivery system could be very useful, because it could be applied early and could produce consistent effects in situ over several months to prevent and inhibit HS formation. However, it is highly crucial to find a proper drug delivery system for GS-Rg3, because it is poorly soluble and shows high crystallinity under physiological conditions [16]. Polymeric drug delivery systems are capable of improving therapeutic efficacy, reducing toxicity and enhancing patient compliance, owing to their controlled delivery of drugs. Electrospinning is a remarkably simple and powerful technique used to generate polymeric fibers at a sub-micrometer scale (ranging from  $\sim$ 50 nm to several micrometers) [17–20]. Biodegradable electrospun poly(L-lactide) (PLA; FDA) has been demonstrated to be a potentially effective carrier for drug delivery [15,21]. In addition to drug delivery, the porous nature of the polymeric mats provides an extremely high surface-to-volume ratio to enable cell attachment, and it contains interconnected sub-micrometer-sized pores that are essential for transporting oxygen and nutrients to support cell growth, which could help promoting wound healing and tissue repairing [22]. Therefore, PLA electrospun membranes are particularly appealing as wound dressings, membranes for tissue repairing and drug delivery systems in the field of biomedical engineering [23,24].

In the present study, GS-Rg3/PLA electrospun fibrous membranes were used to repair wounds in vivo and allow the sustained release of GS-Rg3 to inhibit HS. A further study was then undertaken to investigate the effect of topical application of GS-Rg3-loaded electrospun membranes immediately after trauma on the prevention and reduction of HS in a rabbit ear HS model (Scheme 1).

## 2. Materials and methods

### 2.1. Materials

GS-Rg3 was purchased from Fusheng Pharmaceutical Ltd. (Dalian, China). PLA (Mw = 50 kDa, Mw/Mn = 1.61) was prepared by bulk ring-opening polymerization of L-lactide using stannous chloride as an initiator (Jinan Daigang Co., Jinan, China). 1,1,1,2,2,2-Hexafluoro-2-propanol (HFIP) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). All other chemicals and solvents were of reagent grade and were purchased from Guoyao Regents Company (Shanghai, China).

### 2.2. Electrospinning

GS-Rg3 (20 mg, 60 mg or 100 mg) was dissolved in 2 g HFIP, and 1 g PLA was dissolved in 2.5 g dichloromethane. The electrospinning

solutions were prepared by mixing the GS-Rg3/HFIP solution with the PLA/dichloromethane solution. Three amounts of GS-Rg3 were then incorporated into the PLA fibers (PLA-2%, PLA-6% and PLA-10%) by electrospinning. The electrospinning process was performed as described previously [25]. Briefly, the electrospinning apparatus was equipped with a high-voltage statitron (Tianjin High Voltage Power Supply Co., Tianjin, China) with a maximum voltage of 50 kV. To enable a consistent flow from the capillary outlet, the flow rate of the polymer solution was controlled by a precision pump (Zhejiang University Medical Instrument Co., Hangzhou, China). A grounded aluminum foil was used as a collector. The GS-Rg3/PLA solution was loaded in a 2-ml syringe attached to a cylindrical metal syringe needle, which was used as the nozzle. The voltage for electrospinning was set at 15 kV, and the tip-to-collector distance was fixed at 12 cm. The electrospun membranes were collected on the surface of the aluminum foil and vacuum dried at room temperature for 24 h.

### 2.3. Characterization of electrospun fibrous membranes

The thickness and size of the fibrous membranes were measured using a micrometer, and their apparent density and porosity were calculated according to previously published methods. The morphology of the fibrous membranes was observed using scanning electron microscopy (SEM; FEI Quanta 200, Netherlands). At least five images were taken for each membrane sample, and the fiber diameter of membranes was measured from the SEM images at 5000 $\times$  magnification. At least 20 different fibers and 200 different segments were randomly selected from each image to generate an average fiber diameter using Photoshop for calculations [26].

### 2.4. Rabbit-ear HS model

The fibrotic rabbit ear model of cutaneous scarring was used as described previously, with minor modifications [27,28]. Eighteen New Zealand White rabbits weighing 4.5–5 kg were purchased from the Shanghai Animal Center at the Chinese Academy of Science. The Institutional Review Committee of Shanghai Jiao Tong University, School of Medicine, approved all animal study protocols. For the surgical procedure, animals were anesthetized using an intramuscular injection of ketamine (10 mg kg<sup>-1</sup>) and xylazine (3 mg kg<sup>-1</sup>). Carefully avoiding the central ear artery and marginal ear veins, six full-thickness wounds (down to the cartilage) were created on the ventral side of each ear, using a 10-mm punch biopsy tool. Each two wounds were separated by at least 12 mm. The perichondrial membrane was then dissected off the cartilage. A 2-mm-round-skin undermining was made at the round-margin of each wound to enable the embedding of the electrospun membranes. Occasional bleeding was treated using manual compression. Each wound was covered with a polyurethane Tegaderms dressing (3 M, St. Paul, MN, USA) to prevent interference between treatments. Wounds were kept covered until postoperative day 5.

### 2.5. In vivo release

To evaluate the in vivo release of GS-Rg3, the GS-Rg3/PLA fibrous membranes were transplanted into the rabbit ear HS model using the methods described in Section 2.4. Electrospun fibrous membranes  $\sim$ 800  $\mu$ m thick were cut into round pieces (1.2  $\times$  1.2 cm<sup>2</sup>), which fit the wounds. Prior to use, the pieces were sterilized by electron-beam irradiation (a total dose of 80 cGy) using a linear accelerator. Each electrospun fibrous membrane was transplanted carefully into each wound, and its margin was embedded in the undermined skin. At predetermined time intervals (0, 1, 2, 4, 6, 8, 12 weeks), the total wound area containing the membranes and the wound tissue were harvested for analysis.

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