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Optimization of intrinsic and extrinsic tendon healing through controllable water-soluble mitomycin-C release from electrospun fibers by mediating adhesion-related gene expression

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ABSTRACT

To balance intrinsic and extrinsic healing during tendon repair is challenging in tendon surgery. We hypothesized that by mediating apoptotic gene and collagen synthesis of exogenous fibroblasts, the adhesion formation induced by extrinsic healing could be inhibited. With the maintenance of intrinsic healing, the tendon could be healed with proper function with no adhesion. In this study, we loaded hydrophilic mitomycin-C (MMC) into hyaluronan (HA) hydrosols, which were then encapsulated in poly(L-lactic acid) (PLLA) fibers by micro-sol electrospinning. This strategy successfully provided a controlled release of MMC to inhibit adhesion formations with no detrimental effect on intrinsic healing. We found that micro-sol electrospinning was an effective and facile approach to incorporate and control hydrophilic drug release from hydrophobic polyester fibers. MMC exhibited an initially rapid, and gradually steadier release during 40 days, and the release rates could be tuned by its concentration. *In vitro* studies revealed that low concentrations of MMC could inhibit fibroblast adhesion and proliferation. When lacerate tendons were healed using the MMC-HA loaded PLLA fibers *in vivo*, they exhibited comparable mechanical strength to the naturally healed tendons but with no significant presence of adhesion formation. We further identified the up-regulation of apoptotic protein Bax expression and down-regulation of proteins Bcl2, collage I, collagen III and α-SMA during the healing process associated with minimum adhesion formations. This approach presented here leverages new advances in drug delivery and nanotechnology and offers a promising strategy to balance intrinsic and extrinsic tendon healing through modulating genes associated with fibroblast apoptosis and collagen synthesis.
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1. Introduction

Adhesion formation during tendon healing may cause difficult joint movement and is a major clinical complication. With improvements in surgical techniques and post-operative mobilization, tendon adhesions after tendon repair have been decreased, but the problem has not been solved completely. Many different approaches have been tried to prevent adhesion formation. Physical barriers with biochemical drugs are the main approaches to reduce adhesions [1–5]. However, these drugs either have low efficacy in preventing adhesion formation or have a detrimental effect on the strength of the healed tendon, and the mechanism of how these agents affect the healing response remains unclear [6–9].

To prevent the adhesion formation successfully, we first need to understand the mechanism of tendon healing and adhesion formation. As reported, there are two types of tendon healing: intrinsic and extrinsic. Intrinsic healing is developed by the proliferation and migration of tenocytes from the epitenon and endotenon into the injury site whereas extrinsic healing is achieved by invasion of cells from the surrounding sheath and synovium

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[10,11]. Tendons healed intrinsically exhibit superior biomechanics and fewer dysfunctions while extrinsic healing facilitates formation of scar tissues which may result in adhesion formation, disrupting tendon gliding [12]. It is thus essential to control the excessive scar formation in order to restore the functional integrity of repaired tendon after surgery. So far, some studies have prevented adhesion and scar formation by inducing fibroblast apoptosis and inhibiting collagen secretion [13]. It is therefore expected that inducing fibroblast apoptosis by mediating apoptotic gene and inhibiting collagen expression may effectively prevent adhesion formation.

Mitomycin-C (MMC), an anti-tumor agent, has demonstrated its capability to prevent post-operative adhesion formation by inhibiting fibroblast proliferation and inducing fibroblast apoptosis [14–16], and has been used topically in ophthalmic surgery as well as otorhinolaryngology [14,17]. In addition, MMC mainly induces the apoptosis of fibroblasts by mediating apoptotic genes such as Bcl-2 and Bax [18,19] and also inhibits collagen synthesis in normal dermal fibroblast and HaCaT cells [20]. We hence expect that MMC may reduce adhesion formation by mediating apoptotic gene expression and inhibiting collagen expression in adhesion tissues.

The use of MMC is, however, complicated with much acute and chronic toxicity. For example, significant local tissue damage may occur when it is used in local injections at high concentration, leading to increased scar tissue formation and even failure of wound healing [21]. It may also cause irreversible myelosuppression and hemolytic uremic syndrome with long-term intravenous infusion, limiting its clinical application. Consequently, efforts have been made to reduce the toxic effects of MMC by using various delivery methods. Being water-soluble, MMC can only be dissolved directly in water but not in other organic solvents. Many studies have employed nano- or micro-particles to encapsulate MMC in different delivery systems using various polymers including dextran, N-succinyl-chitosan, albumin, polybutylcy-anoacrylate, and so on [22–28]. These systems, however, have demonstrated the incapability for local administration of MMC due to fast degradation of the drug carriers in the body, leading to high amount of MMC being released within a short time, thus impairing the tendon repair. Therefore, it is critical to develop a new delivery system with controllable MMC release to prevent peritendinous adhesions without impeding the tendon healing.

Electrospinning offers great flexibility for drug delivery applications [29–31]. For example, coaxial [32], emulsion [33] and mixing electrospinning techniques [34] have been applied to fabricate polymer micro- or nanofibers for delivery of various drugs such as anti-inflammatory drugs [35], anti-cancer drugs [36], and proteins [37]. Emulsion and coaxial electrospinning are most frequently used for water-soluble drug delivery because they can form core–shell structure, in which drug is confined in the core and protected by a polymer shell [38]. Though the core–shell structure of fibers can reduce the drug burst release and maintain stable release rate, achieving emulsion and coaxial electrospinning simultaneously is technically demanding due to spinneret complexity, low drug-loading efficiency and potential instability of coaxial flow upon feeding. Hence, it remains challenging to develop a simple and reliable technique for fabricating electrospun polymer micro- or nanofibers loaded with water-soluble drugs.

Poly(L-lactic acid) (PLLA) is a well-known biodegradable and biocompatible polymer. By varying its molecular weight, its degradation time *in vivo* can be tuned from weeks to months. However, due to the hydrophilic nature of MMC, it cannot be directly dissolved in organic solvents such as dichloromethane (DCM), leading to low drug encapsulation efficiency in the PLLA nano/microparticles. Hydrosol nanoparticles, where water is the dispersed phase and possesses high dispersion stability, may be obtained by ultrasonic dispersion and have been used as efficient drug carriers [27]. In the hydrosol nanoparticles, the water-soluble drug can be dissolved into the aqueous solution and readily released in a controlled fashion as a result of free water diffusion through the wall of nanoparticles. Meanwhile, as hydrosol nanoparticles can be dispersed in the organic solvent at various concentrations, the amount of MMC in the organic solvent can thus be controlled. Furthermore, the hydrosols may function as an isolated system that protects the activity of MMC within it.

The aim of this project was therefore to develop a new and efficient micro-sol electrospinning technique to fabricate core–shell polymer fibers for controllable loading and release of hydrophilic MMC to achieve prevention of tendon adhesion formation with no detrimental effect on tendon healing (Scheme 1). In present study, the morphology, diameter, wettability and mechanical strength of the resultant electrospun fibers were first characterized. Release of MMC from the electrospun fibers was subsequently examined. *In vitro* fibroblast behaviors including viability, adhesion, proliferation and apoptosis on the MMC-loaded PLLA fibrous membranes were further evaluated and the *in vivo* tendon healing and formation of adhesion tissue were further investigated using rat Achilles and rabbit flexor digitorum profundus (FDP) tendon models. To understand the mechanism and to control the adhesion formation, expression of adhesion associated Bcl-2, Bax, collagen I, collagen III and α-SMA in the adhesion tissues were finally determined.

2. Materials and methods

2.1. Materials

Fermentation-derived hyaluronan (HA, sodium salt, Mw = 0.5 MDa) without further purification was purchased from Yuancheng Technology Co. (Wuhan, China). PLLA (Mw = 100 kDa, Mw/Mn = 2.16) was obtained from Jinan Daigang Co. (Jinan, China). All other chemical reagents, unless otherwise stated, were purchased from GuoYao Regents Company (Shanghai, China). All tissue culture plastics (TCPs) were purchased from Nunc (Roskilde, Denmark). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin and penicillin/streptomycin were purchased from Gibco (Gibco, Grand Island, NY). Collagen I was from Biorbyt, Cambridge, UK. Collagen III was from Abbiotec, San Diego, CA, USA. α-SMA, Bcl-2 and Bax were from Protein Tech Group, Wuhan, China. β-actin was from Abcam, Cambridge, MA, USA.

2.2. Electrospinning of fibrous membranes

First, 10 mg HA was dissolved in 990 mg distilled water to make 1 wt % HA hydrosols. Then, 10 mg and 40 mg MMC (Kyowa Hakko Kirin, China) were separately mixed into HA hydrosol. Afterwards, a solvent mixture containing 4 g DCM and 1% Span-80 (with respect to PLLA) was mixed with the drug-loaded HA hydrosol, and the mixture was stirred for 20 min to obtain uniform water-in-oil (W/O) emulsions containing micro-sol particles. At last, 2.0 g N, N-dimethylformamide (DMF) and 1.0 g PLLA were dissolved in the emulsion to obtain micro-sol electrospinning solution. The control PLLA solution was obtained by mixing and stirring 1.0 g PLLA, 4.0 g DCM and 2.0 g DMF.

A 0.7 mm diameter needle was fitted to a 2.0 ml glass syringe and a syringe pump. The solutions with 1%, 4% (10 mg, 40 mg) MMC or without MMC were drawn into the syringe to prepare PLLA-MMC1, PLLA-MMC2 and PLLA membranes, respectively. The concentrations of 1 and 4% were optimized to achieve significant difference in the cell inhibition effect with no substantial cytotoxicity (cell viability <50%). A high-voltage power supply provided a

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