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Procyanidins-crosslinked aortic elastin scaffolds with distinctive anti-calcification and biological properties

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ABSTRACT

Elastin, a main component of decellularized extracellular matrices and elastin-containing materials, has been used for tissue engineering applications due to their excellent biocompatibility. However, elastin is easily calcified, leading to the decrease of life span for elastin-based substitutes. How to inhibit the calcification of elastin-based scaffolds, but maintain their good biocompatibility, still remains significantly challenging. Procyanidins (PC) are a type of natural polyphenols with crosslinking ability. To investigate whether pure elastin could be crosslinked by PC with anti-calcification effect, PC was first used to crosslink aortic elastin. Results show that PC can crosslink elastin and effectively inhibit elastin-initiated calcification. Further experiments reveal the possible mechanisms for the anti-calcification of PC crosslinking including (1) inhibiting inflammation cell attachment, and secretion of inflammatory factors such as MMPs and TNF-α, (2) preventing elastin degradation by elastase, and (3) direct inhibition of mineral nucleation in elastin. Moreover, the PC-crosslinked aortic elastin maintains natural structure with high pore volume (1111 μL/g), large pore size (10–300 μm) and high porosity (75.1%) which facilitates recellularization of scaffolds *in vivo*, and displays excellent hemocompatibility, anti-thrombus and anti-inflammatory potential. The advantages of PC-crosslinked porous aortic elastin suggested that it can serve as a promising scaffold for tissue engineering.

1. Introduction

Decellularized extracellular matrix (dECM) has been used clinically as bioprosthesis in the substitution of severe diseased tissues [1]. Recently, due to excellent biocompatibility, dECM has been studied as natural scaffolds for the construction of many tissue engineered organs, such as heart, lung, spleen, liver, pancreas, blood vessel, heart valves, etc.[1–5]. However, based on data from both research experiments and clinical application, dECM is easily calcified on its two major structural components, elastin and collagen, which results in the deterioration and dysfunction of the implanted bioprosthesis when they were clinically used [6,7]. As an importance component of connective tissues, elastin has been used for tissue engineering and regeneration applications in the form of pure and composite biomaterials [8–13]. However, detailed studies revealed that the calcification is initiated by elastin [6,7,14–17], which has been shown to have nucleation sites for apatite minerals, especially when elastin is under degradation in diseased tissues such as heart valve and blood vessels. Therefore, calcification may be further strengthened by inflammation cells adhesion and subsequent secretion of matrix metalloproteinases (MMPs) [6,7,14–21]. Previous studies imply that elastin or elastin-containing biomaterials will inevitably calcify as they gradually degrade during host tissue formation after being implanted either as bioprosthesis or as scaffolds for tissue engineering. Therefore, it is critical for elastin or elastin-containing biomaterials to undergo anti-calcification treatment prior to clinical applications. Glutaraldehyde (GA) is a traditional crosslinking reagent, which has been found to stimulate dECM calcification and introduce cytotoxicity, and is not suitable for crosslinking elastin containing tissues. Bailey et al. [14] has found that the treatment of elastin with aluminum chloride ($AlCl_3$) could protect elastin from further calcification. But the $AlCl_3$ is toxic for the human body, in particular the nervous system. Tripi et al. [22] treated elastin-containing carotid dECM using pentagalloyl glucose yielding crosslinked materials with anti-calcification property. However, whether it could prevent pure elastin from calcification remained unknown, Moreover, the

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anti-calcification effect and related mechanisms of these reagents remained also unknown which may hinder their further clinical application.

Procyanidins (PC) is a type of natural derived polyphenols, widely available from fruits, nuts, vegetables and pine bark [23]. PC has shown antivirus, antibacterial, anticarcinogen and anti-inflammatory bioactivities as well as anti-aggregation of platelets [23,24]. Moreover, PC does not have acute and sub-acute toxicity, and its metabolic ways have been well understood [25]. Previously, studies have shown that PC is able to crosslink heart valve dECM [26–28], and can effectively inhibit calcification of valvular dECM [28]. Since the heart valve dECM contains elastin, it is possible that the crosslinking and anti-calcification effects of PC on dECM might be related to the interaction of PC with elastin. Therefore, we hypothesize that the crosslinking of pure elastin by PC may reduce the risk of elastin-initiated calcification. In this study, PC was firstly used to crosslink pure aortic elastin to investigate the inhibition effect of PC on elastin-initiated calcification of decellularized tissue. Furthermore, the biological properties of PC-crosslinked aortic elastin were evaluated to primarily explore its potential applications as a promising scaffold for tissue engineering.

2. Materials and methods

2.1. Preparation of aortic elastin

Fresh porcine hearts were obtained from a local slaughterhouse and immediately transported to the laboratory in a cold (4 °C) sterile D-Hanks solution, a balanced salt solution without Ca^{2+}, supplemented with 5% penicillin/streptomycin. A 2–3 cm supravalvular segment (10 mm × 10 mm) of ascending aorta was cleared, dissected and washed thoroughly in sterile saline. The aorta segments were firstly decellularized as previously described [26]. Specifically, the fresh porcine aorta was treated with 0.25% trypsin (sigma), 0.02% EDTA (PH7.4) in D-Hanks solutions (10 mL/cm^2) for 30 min at 37 °C, and then extensively incubated in 0.5% triton X-100, 0.5% sodium deoxycholate and 0.02% EDTA for 48 h with gentle shaking at 37 °C. Aortic samples were further rinsed with ribonuclease A (sigma, 20 μg/mL) and deoxyribonuclease (sigma, 0.2 mg/mL) for 2 h to remove cellular components. Additionally, the porous aortic elastin was obtained by cyanogens bromide (CNBr) treatment according to a published method [13]. Briefly, the decellularized aorta segments were soaked in a solution of 70% formic acid containing 50 mg/mL CNBr for 19 h at 20 °C followed by 60 °C for 1 h, and then boiled for 5 min to deactivate CNBr. The obtained porous aortic elastin was washed several times and stored in D-Hanks solution for further use.

2.2. Histological analysis and SEM observation

Fresh aorta segments, decellularized aorta segments and segments of porous elastin were fixed in 4% (v/v) formaldehyde in phosphate buffer (pH 7.2), embedded in paraffin and sectioned to 6-μm thickness slice. Finally, the slices were stained with hematoxylin and eosin (H&E) for histological analysis [15]. In addition, some decellularized aorta segments and segments of porous elastin were fixed in 2.5% glutaraldehyde (GA) and dehydrated and sputtered with gold for scanning electron microscopy (SEM) observation [26].

2.3. Crosslinking of porous aortic elastin with PC

In this study, we used grape seed-extracted PC (Tianjin Jianfeng Natural Product R&D Co., Ltd., with a purity of over 95%) as a tissue crosslinking reagent, which is mainly composed of a dimer form of catechin and/or epicatechin (e.g. catechin–catechin, epicatechin–epicatechin, catechin–epicatechin.). Briefly, porous aortic elastin was crosslinked by soaking in PC solution at concentrations of 0.1, 0.5, 1, 5 mg/mL in D-Hanks solution (pH7.4) at 37 °C using 10 mL/1 cm^2 aorta segment under continuous shaking for 48 h. After crosslinking, the porous aortic elastin was washed several times and photographed using a digital camera. In addition, aortic elastin specimens crosslinked by 6.25 mg/mL (0.625%) GA in D-Hanks solution and treated in same conditions served as control [26]. The cumulative pore volume and pore mean diameter of the porous aortic elastin were determined by using the Hg intrusion porosimetry analysis (Autopore IV 9510 porosimeter) for the characterization of macroporosity [29]. The pore volume (μL/g) of different diameter scope (V_{i-J}) is calculated according to following formula:

$$V_{i-j} = V_j - V_i$$

where i and j are lower and higher pore diameters, respectively. V_i and V_j are the cumulative pore volumes at pore diameters i and j, respectively.

2.4. In vivo anti-calcification analysis

To determine whether PC-crosslinked aortic elastin could inhibit calcification *in vivo*, we evaluated the non-, GA- and PC-crosslinked specimens ($n = 10$) using a Sprague–Dawley (SD) rat subcutaneous implantation model [24,30]. SD rats (SLAC Experimental Animal Co., Shanghai, China) with a body weight of 150 ± 2 g, were housed under a standard facility (filtrated air, 20–25 °C temperature, relative humidity at 50–60%, 12 h light: dark cycle), and had unlimited access to standard diet, water and cleaned air throughout the experimental period. Specimens were implanted subcutaneously on the back of the animals ($n = 5$). On the back of each rat, from the front to the rear, lined non-, GA-, PC (at crosslinking concentration of 0.1 mg/mL)-, PC (at crosslinking concentration of 1 mg/mL)- and PC (at crosslinking concentration of 5 mg/mL)-crosslinked elastin specimens. 21 days later, the specimens were collected and fixed with 4% paraformaldehyde. For calcification analysis, the specimens were embedded in paraffin, sectioned and stained with alizarin red. For quantitative analysis of calcium and phosphorus contents, samples were lyophilized to obtain dry weight (10–15 mg each), and individually hydrolyzed in 1.0 mL of 0.02 N HCl for 12 h. Calcium and phosphorus contents were then measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES; VISTA AX, Varian, USA). All animal experiments were approved by the Shanghai Institute of Life Sciences, Chinese Academy of Sciences.

2.5. Exploration on anti-calcification mechanisms of PC

2.5.1. In vitro macrophage adhesion and TNF-α immunoassay

THP-1 cell line derived from the blood of a patient with acute monocytic leukemia was obtained from the Shanghai Institutes of Biological Sciences and cultivated in RPMI 1640 (Gibco) medium supplemented with 10% FBS (HyClone) and 1% penicillin–streptomycin (Gibco) at 37 °C and 5% CO_2. For differentiation to the monocyte/macrophage phenotype, the cells were induced with RPMI-1640 containing 100 ng/mL phorbol-12-myristate-13-acetate (PMA) (Sigma, Saint Louis, USA) for 48 h, and subsequently resuspended in RPMI 1640 to a final density of 10^6 cells/mL. The cell-seeded specimens were cultured in a humidified atmosphere with 5% CO_2 for 16 h at 37 °C in an incubator. Then the culture medium was collected, centrifuged (4 °C, 10,000 rpm, 5 min) and frozen at −80 °C for further TNF-α assay using human TNF-α immunoassay ELISA Kit (R&D Systems) as described in detail in the manufacturer's protocols [31,32]. The cell-seeded specimens ($n = 3$ per