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# Vascular scaffolds with enhanced antioxidant activity inhibit graft calcification

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ABSTRACT

There is a need for off-the-shelf, small-diameter vascular grafts that are safe and exhibit high long-term patency. Decellularized tissues can potentially be used as vascular grafts; however, thrombogenic and unpredictable remodeling properties such as intimal hyperplasia and calcification are concerns that hinder their clinical use. The objective of this study was to investigate the long-term function and remodeling of extracellular matrix (ECM)-based vascular grafts composited with antioxidant poly(1, 8-octamethylene-citrate-co-cysteine) (POCC) with or without immobilized heparin. Rat aortas were decellularized to create the following vascular grafts: 1) ECM hybridized with POCC (Poly-ECM), 2) Poly-ECM subsequently functionalized with heparin (Poly-ECM-Hep), and 3) non-modified vascular ECM. Grafts were evaluated as interposition grafts in the abdominal aorta of adult rats at three months. All grafts displayed antioxidant activity, were patent, and exhibited minimal intramural cell infiltration with varying degrees of calcification. Areas of calcification co-localized with osteochondrogenic differentiation of vascular smooth muscle cells, lipid peroxidation, oxidized DNA damage, and cell apoptosis, suggesting an important role for oxidative stress in the calcification of grafts. The extent of calcification within grafts was inversely proportional to their antioxidant activity: Poly-ECM-Hep > ECM > Poly-ECM. The incorporation of antioxidants into vascular grafts may be a viable strategy to inhibit degenerative changes.
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## 1. Introduction

There is an urgent need to develop clinically acceptable, readily available, small diameter (<6 mm) vascular grafts for patients with severe vascular disease that do not have suitable veins or arteries for autologous grafts or are not appropriate candidates for placement of intravascular stents. Synthetic vascular grafts fabricated from expanded polytetrafluoroethylene (ePTFE) and polyester (Dacron) are not commonly used to reconstruct small blood vessels due to complications such as thrombosis, stenosis and infection [1,2]. Moreover, synthetic grafts cannot be biologically remodeled in the body or grow with the patient, which is a significant barrier to their use in pediatric cardiac and vascular surgeries [3]. Biological vascular grafts such as allografts (e.g. CryoVein, human saphenous and femoral veins; CryoLife) and xenografts (e.g. Artegraft, bovine carotid arteries; Artegraft Inc) are on-demand, off-the-shelf options; however, clinical trials have not convincingly demonstrated any advantage in patient outcome compared to synthetic grafts [4].

Bioengineered, acellular vascular grafts fabricated from biodegradable synthetic polymers such as polycaprolactone [5], decellularized vascular extracellular matrix (ECM) [6,7], or synthetic polymer/ECM composites [8,9] have been evaluated as alternatives

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to vein and radial artery grafts, which are the current gold standard conduits for lower extremity and cardiac revascularization. Rigorous long-term safety and efficacy assessment *in vivo* must be completed to evaluate time-dependent durability of bioengineered vascular grafts because *in vitro* studies alone or short-term *in vivo* experiments do not capture time-dependent degeneration of the implanted material. Despite this need, very few studies have critically assessed long-term (defined as ≥3 months for small animals [5], ≥12 months for large animals [10] and humans [11]) safety and efficacy of bioengineered vascular grafts *in vivo*. Although common evaluative end-points predominantly focus on graft stenosis due to neointimal hyperplasia or intravascular thrombosis, intra-graft calcification is often overlooked despite its importance in vascular tissues such as biological heart valves [12,13], smooth muscle biology [14], and arterial mineralization in patients with vascular disease, diabetes, and chronic kidney failure (patients likely to be recipients of vascular grafts) [15]. Therefore, intra-graft calcification is a pathology that can lead to vascular graft failure in the long-term.

The detailed mechanisms by which calcifications form within vascular grafts are not known. Nevertheless, several studies highlight the critical role of oxidative stress in the pathology of most forms of arterial mineralization [16,17]. In this study, we hypothesized that improving antioxidant properties of vascular grafts leads to favorable graft remodeling, by reducing tissue and cellular damages (e.g. inflammation, apoptosis, and calcification) caused by oxidative stress. We previously reported the synthesis of antioxidant, citric based polymers [18,19] and the preparation of antioxidant, citric acid-based polymer-ECM composite vascular grafts whereby the polymer coating was functionalized with heparin to enhance graft thromboresistance [8,9]. When compared to vascular ECM grafts, the polymer-ECM composite grafts exhibited reduced neointimal hyperplasia after one month of implantation in the abdominal aorta of Sprague Dawley rats [9]. Herein we report a correlation between the extent of antioxidant activity, oxidative tissue damage, and calcification in vascular grafts. We suggest that manipulating the antioxidant properties of the graft may be a novel strategy to reduce intra-graft calcification.

## 2. Materials and methods

### 2.1. Source of vascular ECM

Male Sprague Dawley rats weighing 200–250 g (Charles River Laboratories, Chicago, IL) were used as tissue (aorta) donors or recipients of vascular grafts. All animal care was performed in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all experiments using animals were approved by the Animal Care and Use Committee of Northwestern University (Chicago, IL). The descending thoracic and abdominal segments of the aorta (3–4 cm in length) were recovered from each donor animal by the Microsurgical Core at Northwestern University (Chicago, IL) as previously described [8].

### 2.2. Preparation of vascular grafts

Three types of ECM-based bioengineered vascular grafts were prepared. Decellularization of rat abdominal aortas was performed with Triton-X, sodium dodecyl sulfate, and Deoxyribonuclease I as described previously by us [8], and used as control group (ECM). Poly (1,8-octamethylene citrate)-*co*-cysteine (POC-Cys) was synthesized with 1,8 octanediol, citric acid and L-cysteine with molar ratio 1:1:0.2, as described previously [20], and hybridized onto decellularized aorta ECM at 45 °C followed by 2-β mercaptoethanol treatment as experimental group 1 (Poly-ECM) [9]. Lastly, the polymer-ECM composite was reacted with N-[β-maleimidopropionic acid] hydrazide (BMPH)-heparin via click chemistry to immobilize heparin onto the Poly-ECM as experimental group 2 (Poly-ECM-Hep) [9].

### 2.3. Antioxidant activity measurements

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Sigma-Aldrich, St Louis, MO) based free-radical scavenging assay was performed to evaluate the antioxidant activity of each graft [19]. Briefly, all three types of grafts (n = 3 per group) were weighed and incubated in a solution of ABTS (7 mM) and sodium persulfate (2.45 mM) at 37 °C. At each time point (days 0, 3, 6 and 9), ABTS solution was sampled, diluted with MQ water 1:10 and the absorbance measured at 734 nm. The antioxidant activity was expressed as percent free-radical scavenging by measuring the decrease in absorbance compared to control ABTS solutions (0% free-radical scavenging), and normalized to the weight of each graft.

### 2.4. Binding of calcium to the grafts

All three types of vascular grafts were incubated in simulated body fluid (SBF) [21] at 37 °C under aseptic conditions, with fresh SBF changed weekly. At 1, 2 and 3 months after incubation, an n = 3 samples from each group was collected, rinsed with PBS and weighed. The samples were then incubated in 0.1 M hydrochloric acid overnight, and assayed for calcium concentration using a calcium colorimetric assay kit (Sigma-Aldrich, St Lois, MO). The results were expressed as calcium content normalized to the mass of each graft.

### 2.5. Implantation and non-invasive imaging of vascular grafts

A rat abdominal aorta interposition model previously described by us [9] was used to evaluate all three types vascular grafts (1 cm in length, n = 4 for each group), using a sutured end to end anastomosis. All vascular grafts were sterilized via ethanol and rehydrated with sterile PBS prior to implantation. All implantation surgeries were conducted at the Northwestern University Microsurgical Core (Chicago, IL). Biweekly vascular ultrasound imaging was performed in accordance with guidelines for noninvasive vascular laboratory testing [22] at the Center for Comparative Medicine (CCM), Northwestern University (Chicago, IL) using an M7/M7T Diagnostic Ultrasound System with an L14-6S probe (Mindray Bio-Medical Electronics, Shenzhen, China). Briefly, animals were anesthetized via isoflurane inhalation (1%–5%) using VetEquip inhalation anesthesia system, and the abdomen around the surgical area was shaved and ultrasound gel was applied. Duplex ultrasound with color Doppler was first performed using the probe to locate the abdominal aorta and the inferior vena cava. B-mode ultrasound was then used to locate the proximal and distal end-to-end anastomosis along the abdominal segment of the vascular graft. Images were acquired with both Doppler and B-mode. Additionally, spectral Doppler waveform analysis was performed at the site of implanted vascular grafts to quantify the velocity of blood flow within the graft.

### 2.6. Tissue collection

Three months after implantation, the animals were anesthetized for terminal blood collection and tissue recovery. Blood was collected from the inferior vena cava of each animal. A sample of anticoagulant whole blood and a sample of separated serum (0.5 ml each) from each animal was sent to the Clinical Pathology lab at Charles River RADS (Wilmington, MA) for a complete blood

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