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Mitigation of diabetes-related complications in implanted collagen and elastin scaffolds using matrix-binding polyphenol

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ABSTRACT

There is a major need for scaffold-based tissue engineered vascular grafts and heart valves with long-term patency and durability to be used in diabetic cardiovascular patients. We hypothesized that diabetes, by virtue of glycoxidation reactions, can directly crosslink implanted scaffolds, drastically altering their properties. In order to investigate the fate of tissue engineered scaffolds in diabetic conditions, we prepared valvular collagen scaffolds and arterial elastin scaffolds by decellularization and implanted them subdermally in diabetic rats. Both types of scaffolds exhibited significant levels of advanced glycation end products (AGEs), chemical crosslinking and stiffening -alterations which are not favorable for cardiovascular tissue engineering. Pre-implantation treatment of collagen and elastin scaffolds with penta-galloyl glucose (PGG), an antioxidant and matrix-binding polyphenol, chemically stabilized the scaffolds, reduced their enzymatic degradation, and protected them from diabetes-related complications by reduction of scaffold-bound AGE levels. PGG-treated scaffolds resisted diabetes-induced crosslinking and stiffening, were protected from calcification, and exhibited controlled remodeling in vivo, thereby supporting future use of diabetes-resistant scaffolds for cardiovascular tissue engineering in patients with diabetes.

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1. Introduction

Diabetes, one of the major risk factors for cardiovascular disease (CVD), is increasing to epidemic proportions worldwide; currently it affects 8% of the world's population and nearly 26 million people in US alone [1]. Hyperglycemia, resulting from the deficiency in insulin secretion (Type 1 diabetes) or insulin resistance (Type 2 diabetes), combined with dyslipidemia, oxidative stress, and inflammation, significantly increases the risk of atherosclerotic vascular disease [2], aortic valve disease [3,4] and cardiomyopathy [5]. Studies have shown that, despite great advances in diagnosis and treatment of CVD, over the last several years diabetic patients have not shared the same decline in coronary artery disease-related mortality as non-diabetic patients [2].

The primary cause of cardiovascular tissue damage occurring in diabetes is the formation of advanced glycation end products (AGEs), which generate irreversible cross-links on long-lived proteins, such as collagen and elastin [6,7]. Glucose and lipid molecules undergo a series of oxidant-induced fragmentation, leading to the formation of short-chain reactive compounds that react with proteins and form AGEs, such as carboxy-methyl lysine (CML) and pentosidine [8]. Malondialdehyde (MDA) is a marker for oxidative stress and a well known by-product of lipid peroxidation [9]. AGEs impair wound healing and induce excessive inflammation [10], fibrosis, and tissue stiffness [11–13]. As a result, the outcome of reparative surgery and tissue transplantation is more problematic in diabetic patients [14].

Tissue engineering holds great promise to treat cardiovascular diseases [15,16]. Significant progress has been made in the field of blood vessel [17–19], heart valve [20,21] and cardiac tissue engineering [22,23]. It is critical that replacements for damaged cardiovascular structures possess appropriate biomechanical properties from the outset of implantation. Therefore, there is increased interest in collagen and elastin-based biological scaffolds derived from xenogeneic or allogeneic extracellular matrices (ECM), which have optimal physical properties. Furthermore, the 3D structure of the ECM can be preserved with an optimal decellularization technique that removes cells without damaging the

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matrix components [24–26]. Ideally, basement membrane proteins are also retained, as their presence is essential to tissue regeneration [27].

For pre-clinical evaluation, tissue engineered constructs and their remodeling are typically tested in healthy animals [28–30]. However, there are great expectations that TE and regenerative medicine research will offer solutions for patients affected by the cardiovascular complications of diabetes. The complex glycoxidative environment could affect tissue remodeling since the ECM proteins, especially collagen and elastin as well as the matrix metalloproteinases (MMPs) involved in matrix remodeling, might be modified by the formation of AGEs. Matrix alterations that result in activation of inflammation, fibrosis, and impaired healing might not be conducive to the desired integration and remodeling of tissue engineered constructs. These aspects can only be assessed in diabetic animal models with very strict glycemic control [31–33].

Polyphenols, which are based on gallic acid units bound to a polyol core exhibit high affinity for proline-rich proteins [34], particularly to collagen [35] and elastin [36,37]. Penta-galloyl glucose, (PGG) a well-characterized polyphenol [38], can increase the stability of collagen and elastic scaffolds and slow down their degradation [25,35]. PGG has been reported to have many beneficial effects such as antioxidant, antidiabetic, and anti-inflammatory activities [39].

We hypothesized that AGEs could alter the properties of matrix-derived scaffolds, such as collagen scaffolds used for heart valve tissue engineering and elastin scaffolds for blood vessel tissue engineering. This could affect the outcome of tissue engineering products based on biological scaffolds. Polyphenols, by virtue of their antioxidant properties and collagen- and elastin-binding abilities, might protect the structural matrix proteins from diabetes-related complications. Therefore, we used a diabetic rat model and implanted PGG-treated scaffolds subdermally for four weeks. Scaffolds were then removed and analyzed for their mechanical and biochemical properties.

2. Materials and methods

2.1. Materials

High-purity penta-galloyl glucose was a generous gift from N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium (www.omnichem.be). Streptozotocin was from Sigma (S0130). The insulin preparation used for rats in this study was Humulin N U-100 NPH, Human Insulin of rDNA origin Isophane suspension from Lilly (Indianapolis, IN). Electrophoresis apparatus, chemicals, and molecular weight standards were from Bio-Rad (Hercules, CA). Bicinchoninic acid protein assay kit was from Pierce Biotech (Rockford, IL). The Vectastain Elite kit and the ABC diaminobenzidine tetrahydrochloride peroxidase substrate kit were purchased from Vector Laboratories (Burlingame, CA). We used the following antibodies: rabbit anti-collagen IV (Abcam, #ab6586), rabbit anti-laminin (Abcam, #ab11575), monoclonal anti- N-epsilon-(carboxymethyl)lysine (CML) antibody (MAB3247, R&D Systems), monoclonal anti-vimentin (V5255, Sigma), mouse anti-CD8 (GTX76218, GeneTex Inc, Irvine, CA), mouse anti-CD68 anti-macrophage/monocyte antibody, clone ED-1 (MAB1435, Millipore, Billerica, MA). Deoxyribonuclease I was from Worthington Biochemical Corporation (Lakewood, NJ). AlphaTRAK (Gen II) test strip and the AlphaTRAK® Blood Glucose Monitoring System was from Abbott Laboratories, Animal Health (Abbott Park, IL). All other chemicals were of highest purity available and were obtained from Sigma–Aldrich Corporation (Lakewood, NJ).

2.2. Heart valve collagen scaffold preparation

Collagen scaffolds were prepared following a protocol described previously with minor modifications [26]. Briefly, fresh porcine aortic roots were harvested from a local slaughterhouse, cleaned over ice, and placed in double-distilled water overnight at 4 °C to induce hypotonic shock and cell lysis. Next, for complete cell removal, the valves were placed on an orbital shaker at room temperature and treated with 0.05M NaOH for 2 h followed by 70% ethanol for 20 min and an overnight incubation in a mixture of detergents: 0.5% sodium dodecyl sulfate, 0.5% Triton X-100, 0.5% deoxycholate, 0.2% ethylenediaminetetra-acetic acid in 50 mM TRIS, pH7.5. After rinsing five times with double-distilled water and 70% ethanol to remove detergents, valves were treated with deoxyribonuclease/ribonuclease mixture (360mU/ml for each enzyme) for 2 days at 37 °C, to complete the removal of nucleic acids. After rinsing with double-distilled water, valves were sterilized in 70% ethanol overnight at room temperature. Under sterile conditions, the aortic cusps were dissected away from the aortic wall and stored in sterile ddH_20 with 1% antibiotic/antimycotic (Pen-Strep) at 4 °C. Each individual cusp served as a collagen scaffold. This decellularization method effectively removed cells, while preserving valve matrix components and eliminating the porcine α–Gal epitope [26].

2.3. Arterial elastin scaffold preparation

Elastin scaffolds were prepared following an alkaline extraction protocol described before, with minor modifications [25]. Briefly, fresh porcine carotid arteries (60–80 mm long, 5–6 mm in diameter) obtained from Animal Technologies, Inc. (Tyler, TX) were rendered acellular by incubation in 0.1M NaOH solution at 37 °C for 24 h followed by extensive rinsing with deionized water until pH dropped to neutral. Scaffolds were then rinsed and stored in sterile PBS. This treatment removed all cells and most of the collagen, leaving vascular elastin intact. Scaffolds were also completely devoid of the α–Gal epitope (data not shown).

2.4. PGG-treatment of scaffolds

The acellular scaffolds were treated with sterile 0.1% PGG in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution in saline (pH 5.5) containing 20% isopropanol overnight at room temperature under agitation and protected from light. Scaffolds were then rinsed in sterile PBS and stored in sterile PBS containing 1% protease inhibitor and 1% Pen-Strep at 4 °C. The efficiency of PGG binding and tissue stabilization was assessed previously by testing tissue resistance to collagenase and elastase digestion [25,26]. Untreated acellular scaffolds were used as controls.

2.5. Rat model of STZ-induced diabetes

Adult male Sprague–Dawley rats ($n = 40$, weight 300–350 g) were rendered diabetic via a single dose of sterile filtered 55 mg/kg streptozotocin solution in 0.1M citrate buffer (pH 5) by tail vein injection. Control rats ($n = 40$) received an equal volume of vehicle (sterile citrate buffer). Starting on day 3, levels of blood glucose were determined 3–4 times per week, using AlphaTRAK (Gen II) test strips on the AlphaTRAK Blood Glucose Monitoring System, designed specifically for animals. Diabetes was established (>400 mg glucose/dL blood), and diabetic rats were given subcutaneous injections of long-lasting insulin (2-4U Isophane) every other day to maintain blood glucose level in a desirable range (400–600 mg glucose/dL blood) and prevent development of ketonuria and weight loss. Glucose levels, individual weights, hydration status, and food and water consumption were monitored closely and continuously graphed to ensure adequate health parameters. Animals were provided with food and water *ad libitum* and were cared for by the attending university veterinarian and associated staff at the Godley-Snell Research Center animal facility. The Animal Research Committee at Clemson University approved the animal protocol, and National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication #86-23 Rev. 1996) were observed throughout the experiment.

2.6. Experimental implant groups

Scaffolds were divided into four groups as follows: a) non-treated collagen scaffolds; b) PGG-treated collagen scaffolds; c) non-treated elastin scaffolds; d) PGG-treated elastin scaffolds. Samples from each group were implanted subdermally into control and diabetic rats ($n = 20$ implants per group) as detailed below.

2.7. Subdermal implantation

Four weeks after STZ administration, rats were prepped for surgery and anesthetized using 1–2% Isoflurane. A small, transverse incision was made on the back of the rats, and two subdermal pouches were created by blunt dissection. The acellular scaffolds were implanted – one whole acellular aortic cusp (collagen scaffold) in each pocket ($n = 2$ implants per rat), and the incision was closed with surgical staples. Acellular arteries (elastin scaffolds) were cut open longitudinally and 1×3 cm samples were implanted subdermally, as described above for cusps ($n = 2$ per rat). Diabetic rats were given 1U of insulin pre-operatively. The rats were allowed to recover, provided with food and water *ad libitum*, and were cared for by the attending veterinarian and associated staff at the Godley-Snell Research Center animal facility. Post-operative levels of blood glucose were determined 3–4 times per week, and diabetic rats were given insulin as described above. After four weeks, the rats were humanely euthanized by CO_2 asphyxiation and the scaffolds explanted and collected according to their respective assay application as follows: scaffolds for histological analysis were placed in Karnovksy's Fixative (2.5% glutaraldehyde, 2% formalin, 0.1M cacodylic acid, pH 7.4) and paraffin embedded; samples designated for mechanical analysis were collected in sterile PBS with 0.02% NaN_3; and samples for protein, calcium and AGE analysis were flash frozen in liquid nitrogen and kept on dry ice until transferred to −20 °C for storage.