Acta Biomaterialia 10 (2014) 1897–1906

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Dose- and time-dependent effects of genipin crosslinking on cell viability and tissue mechanics – Toward clinical application for tendon repair

Gion Fessel [a,b,1], Jennifer Cadby [a,b,c,1], Stefania Wunderli [a,b], René van Weeren [c], Jess G. Snedeker [a,b,*]

[a] Department of Orthopedics, Balgrist University Hospital, University of Zurich, Balgrist, Forchstrasse 340, CH-8008 Zurich, Switzerland
[b] Institute for Biomechanics, ETH Zurich, Wolfgang Paulistrasse 10, CH-8092 Zurich, Switzerland
[c] Department of Equine Sciences, Faculty of Veterinary Medicine, University of Utrecht, Yalelaan 112, 3584 Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form 21 November 2013
Accepted 18 December 2013
Available online 31 December 2013

Keywords:
Cytotoxicity
Collagen
Tenocytes
Tendon injury
Tendon tear

ABSTRACT

The crosslinking agent genipin is increasingly invoked for the mechanical augmentation of collagen tissues and implants, and has previously been demonstrated to arrest mechanical damage accumulation in various tissues. This study established an in vitro dose–response baseline for the effects of genipin treatment on tendon cells and their matrix, with a view to in vivo application to the repair of partial tendon tears. Regression models based on a broad range of experimental data were used to delineate the range of concentrations that are likely to achieve functionally effective crosslinking, and predict the corresponding degree of cell loss and diminished metabolic activity that can be expected. On these data, it was concluded that rapid mechanical augmentation of tissue properties can only be achieved by accepting some degree of cytotoxicity, yet that post-treatment cell survival may be adequate to eventually repopulate and stabilize the tissue. On this basis, development of delivery strategies and subsequent in vivo study seems warranted.

1. Introduction

Crosslinking has long been employed to augment the mechanical properties of collagen-based implants for the repair or replacement of musculoskeletal and cardiovascular tissues [1–3]. The physiological environments of these systems can expose implants to extreme physical demands that include high mechanical stresses, high mechanical strains and/or highly repetitive loading. Such loading regimes can overwhelm even native tissues, a fact that is evidenced by high clinical rates of connective tissue disease and injury [4].

Although tissue and biomaterial crosslinking strategies, traditionally using glutaraldehyde, have almost exclusively focused on ex vivo chemical treatments of an implant prior to its application, in vivo exogenous crosslinking has more recently been pursued (as we recently reviewed in detail [5]). In this paradigm, the collagen matrix of injured tissue is bolstered by judicious and targeted application of low-toxicity crosslinkers. The idea here is to augment a tissue at the margins of a damaged region, arrest mechanically driven tissue degeneration and possibly provide a foothold for eventual recovery of tissue homeostasis. The use of ultraviolet radiation (in combination with riboflavin as a photosensitizer) to augment the biomechanical properties of connective tissues within the eye [6] has by now become a common clinical treatment of keratoconus [7], a disorder where local matrix weakness leads to tissue bulging under ocular pressure. Proof of concept studies using low toxicity crosslinkers in orthopedic applications are also emerging [8,9].

Of the known low-toxic collagen crosslinking agents, one of the best characterized is genipin (GEN), a naturally occurring organic compound derived from the fruit of the gardenia plant (*Gardenia jasminoides*). At acidic and neutral pH, GEN reacts with primary amines of biopolymers and forms mono- up to tetramer crosslinks [10]. With increasingly basic conditions, GEN further undergoes ring-opening self-polymerization with increasing polymer length prior to binding to primary amines [11]. With increasing polymer length (~4–88-mers), amine reactions with GEN slows, in turn leading to less reduced enzyme digestibility and swelling by GEN [12]. The feasibility and benefit of employing GEN as an alternative to higher toxicity crosslinkers like glutaraldehyde has been

* Corresponding author at: Department of Orthopedics, Balgrist University Hospital, University of Zurich, Balgrist, Forchstrasse 340, CH-8008 Zurich, Switzerland. Tel.: +41 44 386 3755.
E-mail address: jsnedeker@research.balgrist.ch (J.G. Snedeker).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.actbio.2013.12.048
1742-7061/

demonstrated in a range of applications, including heart valves [13], pericardial patches [14], conduits for nerve growth guidance [15], scaffolds for tissue-engineered cartilage [16] and decellularized tracheal transplantation [17], and as a more general application to augment the strength and degradation properties of collagen-based gels [15,18–21].

Our own efforts have demonstrated in an in vitro model that application of GEN can arrest the progression of tendon lesions that are characteristic of acute injury [9], and could potentially be of benefit in addressing this urgent and unmet clinical need (see Ref. [22] for background on tendon tear prevalence and clinical outcome). Tendon injuries are also widespread in equine athletes, with much pathophysiological similarity to tendon injury in man [23]. Using equine tendon, we demonstrated that immersion in a high concentration GEN solution (20 mM for 3 days) could significantly recover post-injury tendon function, bringing it to a level similar to that of uninjured controls. This functional recovery was reflected in reduced tissue strains at a given mechanical stress, increased tissue elasticity and the arrest of mechanical damage accumulation during high-cycle dynamic loading. Although functional efficacy of these GEN treatments was clearly demonstrated, the physiological implications (e.g. cell toxicity) of the treatment were not investigated. More specifically, it was unclear what effect GEN treatment has on resident cell populations, and whether GEN concentrations at levels reported by others as non-cytotoxic [16,24–28] could be sufficient to elicit recovery of mechanical integrity. This information is critical to guide further development of GEN based clinical approaches to in situ tissue augmentation of dense collagen-based connective tissues, including tendon. This information is also necessary to guide the design and development of delivery systems that can provide targeted (local) tissue augmentation without unacceptable collateral damage to peripheral tissues.

The first aim of the present study was to investigate in vitro dose-dependent tendon cell toxicity, exploring effects of both treatment time and concentration. Previous studies have similarly investigated a range of other cell types [16,24–28], with variable results indicating that tissue specific investigation of relevant cell phenotypes is warranted. The present series of studies focus on tenocytes as a class of fibroblastic cells derived from dense collagen connective tissue that has not yet been investigated. The second aim was to investigate the functional effects of GEN treatment on tendon explants to establish dependency of these effects on treatment concentration and duration. Ultimately our goal was to determine whether a balance between non-cytotoxicity and functional (biomechanical) efficacy of GEN dosing could be achieved, widening the potential range of viable clinical applications for this increasingly used collagen crosslinking agent.

2. Method

2.1. Study design

All studies were carried out on isolated cells or tissue explants of the superficial digital flexor tendon (SDFT) from the front limbs of freshly slaughtered horses collected from a local abattoir. All experimental factors (treatment time and concentration) were generally performed with tissue extracted from the same animal and then replicated using tissue from additional animals. Samples were subjected in a random manner to either sham-treatment (incubated in genipin-free cell expansion medium) or in medium supplemented with genipin at concentrations (c_{GEN}) ranging from 0.02 to 20 mM. Incubation times of 24, 72 and 144 h were investigated.

The experiments were conducted starting with a broad approach and progressively focusing on a more limited range of dosages and their effects. First, cell-culture experiments were performed to assess cytotoxicity in terms of relative cell viability and metabolic activity. Using tissue explants, penetration of the crosslinking agent was assessed, homogeneity of crosslink distribution was quantified by inherent fluorescence of GEN crosslinks and the physical effects of the treatments were characterized as changes in denaturation temperature. All these experiments were performed over a wide range of concentrations and treatment times, aiming to identify dosing regimes capable of altering the physical properties of collagen with minimal cytotoxicity. In a second phase, gene expression and cell motility were examined within a reduced range of dosing regimes. Finally, tissue mechanics were characterized for a targeted range of GEN dosing, to identify minimal dosing thresholds able to achieve functionally relevant changes in biomechanical properties.

2.2. Isolation of cells and tissue explants

Tissue explants were dissected from the core of the SDFT to a standardized size of approximately $2 \times 2 \times 2\ mm^3$ under sterile conditions using previously described dissection methods [9], then incubated in either GEN-supplemented or control medium. For isolated cell-culture experiments, tendon cells were extracted by digestion of explanted tissue using protease type XIV (Sigma–Aldrich, St. Louis, MO) for 2 h at 37 °C and collagenase B solution (Roche, Burgess Hill, UK) for 16 h at 37 °C. After the digestion process, the mixture was filtered and centrifuged. The cell pellet was resuspended, seeded at a density of 10^4 cells cm^{-2}, then cultured at 37 °C and 5% CO_2 in expansion medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum, 50 μg ml^{-1} gentamicin and 1.5 μg ml^{-1} fungizone, all from Life Technologies, Paisley, UK). Cells used in experiments were either freshly digested or passaged once at subconfluency.

2.3. Genipin crosslinking

A 20 mM stock solution of GEN (Challenge Bioproducts Co., Taiwan) was freshly prepared in expansion medium for each experiment and then sterile filtered (Millipore, Carrightwohill, Cork, Ireland). The stock was then diluted to the required concentrations. For explant cell viability and differential scanning calorimetry (DSC), explants were incubated in culture dishes containing GEN-supplemented medium at 37 °C and 5% CO_2; for tissue mechanics, explants were incubated in Falcon tubes. Explants used for biochemical analysis and crosslinking distribution were snap frozen after treatment and stored at −80 °C until later use.

2.4. Penetration and color changes

After GEN treatment, excess treatment solution was removed by blotting the samples on clean cellulose tissue. Superficial formation of blue pigmentation that qualitatively indicates GEN crosslinking [29] was documented using a digital camera under consistent illumination. The same samples were then embedded in paraffin according to standard methods and cut into 6 μm sections (RM2265, Leica, Wetzlar, Germany). Inherent sample fluorescence (excitation wavelength (λ_{ex}): 510–560 nm; emission wavelength (λ_{em}): 590 nm) was measured using a fluorescence-equipped upright microscope (Nikon Eclipse E600), since GEN crosslinks have been shown to emit fluorescence, with an exponential correlation to mechanical properties (as measured by storage modulus in collagen gels) [30].