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

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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

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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].

2.5. Cell-culture viability and metabolic activity

Cell viability and metabolic activity was compared to a sham/positive control group (cells incubated in expansion medium only) and to a negative control group (cells were killed with 70% methanol for 1 h prior to the assay). For both tests the experiment was replicated with cells from three tendons of different animals (biological replication), with quadruple repeated measures per experimental condition (technical replication). Cell viability was measured using a viability/cytotoxicity assay (LIVE/DEAD® Viability/Cytotoxicity Kit, Molecular Probes, Life Technologies, Paisley, UK) according to the manufacturer's recommendations. Briefly viability was measured as relative fluorescence ($\lambda_{ex}/\lambda_{em}$: 485 nm/530 nm) in a microplate reader (SpectraMAX Gemini XS, Molecular Devices, Sunnyvale, CA) compared to controls. Similarly, cell metabolic activity was measured as fluorescence ($\lambda_{ex}/\lambda_{em}$: 560 nm/590 nm) after 8 h of incubation in 10% alamarBlue (alamarBlue assay, Invitrogen), as previously described [31]. The control experiment showed negligible influence of GEN light absorption characteristics on these assays. Finally, intensities were converted to cell viability (%) and metabolic activity (%):

$$\frac{F(\lambda_{em})GEN - F(\lambda_{em})C-}{F(\lambda_{em})C+, -F(\lambda_{em})C-} \quad (1)$$

where $F(\lambda_{em})$ represents the average fluorescence intensity of technical replications for each experimental condition at the wavelength (λ_{em}) for each test, with C+ and C− representing the positive and negative controls, respectively.

2.6. Cell motility

Cell motility was determined using a standard scratch assay. Briefly, confluent cell monolayers from three different animals were treated in GEN or control medium for 3 days. The monolayers were then scored with a sterile pipette microtip to leave a scratch of approximately 0.5 mm width. The scratch widths were monitored under an inverted microscope (Zeiss Observer Z1) over 9 h, with digital images collected at 3 h intervals. The scratch width was then measured (Image J software 1.44p, National Institute of Health, USA). The cell migration speed ($\mu m\ h^{-1}$) was calculated at 3, 6 and 9 h after the scratch, and the average cell velocity was determined as the rate of scratch width closure divided by 2 to account for cell movement on each side of scratch.

2.7. Cell viability in explants

Equally sized tendon explants from five horses were weighed (116 ± 12.3 mg) and GEN treated. After 3 days the cells were isolated from the tendon explants using enzymatic digestion as described above and the surviving cells were counted twice in a Neubauer chamber using Trypan blue (Molecular Probes, Life Technologies). Viable cell density was calculated by normalizing the average number of living cells to the initial wet weight of the explants.

2.8. Differential scanning calorimetry in explants

Explants from five animals were treated over the full range of concentrations and incubation times. After briefly blotting with paper to remove excess moisture, they were wet weighed and placed with the largest flat area onto the bottom of stainless steel pans to guarantee optimal heat transfer. After calibration of the differential scanning calorimeter (2920 calorimeter, TA Instruments, Delaware US), the pans were sealed and ramped at a constant heat rate of 10 °C min^{-1} from 0 to 120 °C using an empty pan as the reference. Denaturation was determined as the temperature at the peak heat flow in the endotherm (t_m). As enthalpy has been previously shown to be insensitive to exogenous collagen crosslinking, it was not considered in the analysis [32]. Some pans were opened following DSC measurements, and the samples were dried in an oven for 16 h at 130 °C to determine the dry weight, which was then considered with the wet weight to determine water content [33].

2.9. Gene expression analysis

Cells from eight animals were cultured in a reduced range of concentrations (c_{GEN}: 0, 0.1 and 1 mM) for 3 days at 37 °C and 5% CO_2. At harvesting, cell cultures were resuspended in RNA-Bee™ (TEL-TEST, Friendswood, TX, USA). RNA was precipitated and reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). As markers of matrix metabolism, we assayed the genes for collagen type 1 and matrix metalloproteinase 1, as well as the apoptotic marker caspase 3 (Table 1). As an internal control, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase was used after verification that it was stably expressed across sample conditions (data not shown). Amplifications were performed in duplicates for the tested genes and quadruple for internal controls using a Power Sybr® green polymerase chain reaction (PCR) master mix (Molecular Probes, Life Technologies) according to standard manufacturer guidelines. Quantitative real-time PCR was performed (StepOnePlus™, Applied Biosystems, Life Technologies, Paisley, UK) and analyzed using the device software described method. Averaged gene expressions relative to the housekeeping gene were calculated according to the $2^{-\Delta CT}$ method and used to discriminate between groups [34].

2.10. Mechanical testing

Uniaxial tensile testing was performed with small modifications to previously described protocols, where the power and effectiveness of the employed experimental setup were reported [9,35,36]. Due to the large quantities of tissue needed for mechanical tests, tendons were collected and frozen until the day of testing/treatment. At the start of the experiment, tendons were thawed for 1 h at room temperature. During sample processing

Table 1
Horse specific primers: nucleotide sequences of the tested genes.

Gene	Accession No.	Product length (bp)	Primers Sequences (5′ → 3′)
Casp 3 (Caspase 3)	*NM_001163961*	127	F: TCA-GGC-CTG-CCG-AGG-TAC-AGA R: ACC-AGG-TGC-TGT-GGA-ATA-CGC-A
Col1A1 (collagen IA1)	*XM_001499586*	164	F: TGC-CAT-CAA-AGT-CTT-CTG-CAA R: CGC-CAT-ACT-CGA-ACT-GGA-ATC
MMP-1 (matrix metalloproteinase-1)	*NM_001081847*	92	F: CGA-AGG-GAA-CCC-TCG-GTG-GGA R: TGG-CCT-GGT-CCA-CAT-CTG-CTC
GAPDH (housekeeping gene)	*NM_001163856*	141	F: GTC-AAC-GGA-TTT-GGT-CGT-ATT-GGG R: TGC-CAT-GGG-TGG-AAT-CAT-ATT-GG

and tensile testing, dehydration of the samples was avoided by covering them with sterile gauze soaked in phosphate-buffered saline (PBS), or by spraying them with PBS such that a liquid film was visible on the samples. All of the sample processing and tensile testing was performed at room temperature. Single strips of tendon were subsectioned into either triplets or pairs (triplet = 13, pairs = 11), each of approximately $1 \times 3 \times 50\ mm^3$, using a hand-held device with three microtome blades aligned in parallel (high profile, PTFE coated, 7310, DuraEdge) [9]. All samples were free from the surrounding connective tissue (epitenon). Some resident collagen fibers were likely disrupted, leading to lower values of stiffness and strength compared to whole tendon mechanical properties. Strips from each triplet/pair were then pseudo-randomly allocated either to the control or to one of the GEN treatment groups. The precise cross-sectional area of each specimen was assessed by averaging triplicate readings from a CCD-based custom linear laser scanner (accuracy: $-3.52 \pm 1.89\%$, precision: 0.83%; for details see supplementary materials S-1 and S-1 Fig. 1) adapted from the system described by Vergari et al. [37]. On average, cross-sectional area was $3.9 \pm 0.7 mm^2$, with no differences between groups (analysis of variance (ANOVA), $p = 0.952$). Samples were then mounted in custom clamps after being wrapped in two saline soaked pieces of cloth to reduce slippage and clamping damage [35]. Samples were preconditioned 10 times up to 6 MPa, corresponding to the end of the heel region and the onset of the linear region of the material curve. Upon preconditioning, the stress–strain curves became congruent within 10 cycles, without any apparent damage. After the 11th cycle to an applied stress of 6 MPa, the sample was held stretched and allowed to relax for 300 s (relaxation experiment). Relaxation was simply assessed as the relative stress decay over time, and the end value was used for statistical analysis. Displacement measurements were normalized to nominal strain based on the initial length (L_0) at a tare stress corresponding to 0.05 MPa. After a recovery time again of 300 s, samples were ramped to failure at a constant strain rate of 0.5% $L_0\ s^{-1}$, the strain rate used in all tests (Fig. 1). The nominal stress was calculated based on initial cross-sectional area before treatment. The elastic mechanical properties were measured as the tangential elastic modulus in the linear part of the stress–strain curve. The region of the 20% highest values of the numerical gradient of the stress–strain curve was used to define the linear range. First-order polynomials were fitted to the linear range, with the slope being the tangential elastic modulus. The linear fit was then shifted by 0.2% strain to determine the yield point as the intersection of the stress–strain curve with the linear fit. Maximal stress and corresponding strain (ultimate strength) and strain energy densities up to the yield point were calculated numerically [33].

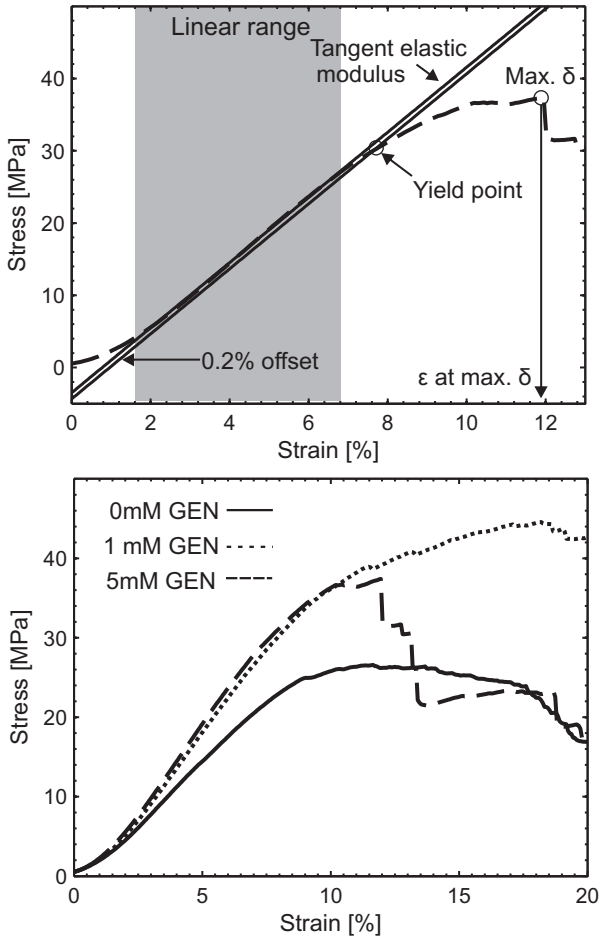

Fig. 1. (Top) Schematic description of parameters used to analyze mechanical data. First a linear range was defined, which includes the region of the stress–strain curve with maximal numerical gradient (the 20% highest values: region shaded in gray). A line was fitted to this linear range, with the slope taken as the tangential elastic modulus. The intersection of the stress–strain curve with a shifted regression line (offset of 0.2%) defined the yield point. The maximum stress (σ) and corresponding strain (ε) were analyzed, as indicated. (Bottom) Sample stress–strain curves from a triple of tendon strips cut from the same tendon.

2.11. Statistical analysis

Normalized outcomes from cytotoxicity experiments (here:% cell viability/metabolism/motility of 100% for controls) were analyzed using probit regression (factor: treatment time; covariate: dose), since this model fits the sigmoidal dose–response curves of toxicity experiments well. A probit regression fits dichotomous/binominal dependent variables (e.g. cells being dead/alive) and is commonly used in toxicology to estimate lethal doses (e.g. LD_{50}). The sigmoid dose–response data (dependent variable) is transformed to become continuous, not limited by 0 and 1, and linear, and can then be analyzed similarly to a linear regression. The probit model was used to estimate toxicity (potency) together with 95% confidence intervals of the GEN treatment in terms of relative effects on cells (similar to LD_{50}). In case there was a common slope for the regression lines for all treatment times (χ^2-parallelism test, $p < 0.05$), axes intercepts and relative potencies were used to assess the effect of treatment duration on cells, after having assessed an adequate model fit (Pearson's goodness-of-fit χ^2-tests: $p > 0.15$). The response of the DSC (t_m) was assessed by analysis of covariance (ANCOVA, fixed factor: treatment time; covariate: concentration), yielding regression lines for each treatment time. In case there was no significant difference of the interaction term (indicating differences of the slopes of regression lines/covariate), the model was additionally reduced to a one-way ANOVA to assess significant effects of concentration (fixed factor: concentration). Post hoc pairwise comparison was performed using Bonferroni correction. Cell motility and mechanical properties were assessed by two-way ANOVAs for randomized block designs (random factor: horse; fixed factor: treatment) and post hoc pairwise comparison was used to discriminate between treatment group effects using Bonferroni correction or, in some cases, Dunnett's test to compare test groups against controls. In case the model assumptions (sphericity) were not met, Greenhouse–Geisser adjustment or the multivariate analysis of variance (MANOVA) approach was used. Gene expression was assessed by Friedman's test, the non-parametric alternative to the blocked ANOVA. In all statistical tests, differences were deemed significant with $p \leqslant 0.05$ and were deemed trends with $p \leqslant 0.1$. In all cases, two-sided tests were performed. Results are reported as means with standard deviations, if not stated otherwise. All statistics were performed using SPSS v21.0 (IBM Corporation, New York, NY) and/or Matlab R2013a (MathWorks, Natick, MA).

3. Results

With increasing GEN concentration and time of incubation, the color of the tendon explants changed from light to dark blue, indicating a reaction of GEN with primary amines (Fig. 2, top).

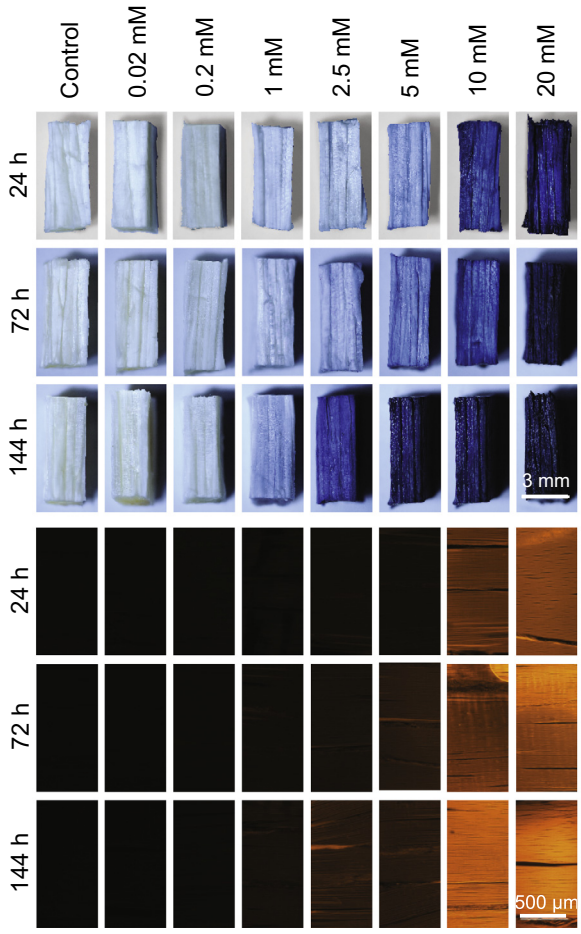

Fig. 2. (Top) Discoloration of incubated tendon explants. A blue pigment is formed when GEN crosslinks primary amines [29]. (Bottom) Inherent fluorescence indicates GEN crosslinks (λ_{ex}: 510–560 nm, λ_{em}: 590 nm) [30] that are formed homogeneously throughout the tendon explants.

Consistent with the dose- and time-dependent discoloration, homogeneous crosslinking was indicated by uniform fluorescence throughout the cut sections (Fig. 2, bottom).

The probit model indicated that cell viability was clearly concentration dependent and that cell viability was reduced in the 144 h treatment group; in other words, the 144 h GEN treatment was more potent/toxic. This can be seen by a significant leftward shift (toward lower concentrations) of the regression line and an increased potency for the 144 h group (details can be seen in Fig. 4, left, and Tables 2–4). While cell rounding was noted at intermediate concentrations, the two highest tested GEN concentrations (10 and 20 mM) apparently fixed the (dead) cell morphology in an elongated state, which is normal for tenocytes. Fluorescence observed throughout the cells may have indicated GEN crosslinking of cellular and intracellular proteins (Fig. 4).

Metabolic activity was also clearly affected in a concentration-dependent manner, coupled with a non-significant reduction of metabolic activity in the 144 h treatment group. These effects can be seen as a non-significant leftward shift of the regression line and a non-significantly increased potency of GEN (Fig. 3, middle, and Tables 2–4).

Cell viability in tendon explants was similarly concentration dependent according to the probit model. However, cell survival was not reduced in the 144 h treatment group, with no significantly shifted curves and no significant reduction in treatment effect (Fig. 3, right, and Tables 2–4).

Two-way ANOVA showed a significant effect of c_{GEN} on migration speed, with no variation over time after scratch application (Fig. 5 and Tables 3 and 4). Post hoc pairwise comparison indicated significantly reduced migration speeds in 1 mM and higher c_{GEN}. ANCOVA revealed a common slope of the regression model fitted to the denaturation temperature, which indicated insuffuciency of treatment duration (interaction term, $p = 0.16$). Therefore, the ANCOVA was reduced to a one-way ANOVA that was dependent on dose only. This showed a significant effect of concentration with increasing denaturation temperature from 5 mM up (Fig. 6 and Table 4).

Friedman's test revealed a significant effect of the GEN treatment on collagen type 1 gene expression. An 83% reduction in the relative gene expression ($2^{-\Delta CT}$) was observed in the 1 mM GEN group compared to the 0 mM GEN controls, as was a trend of 23% decreased relative expression in the 0.1 mM GEN group (Fig. 7 and Table 4). The cell apoptosis marker caspase 3 and MMP 1 both remained unaffected over the tested range of

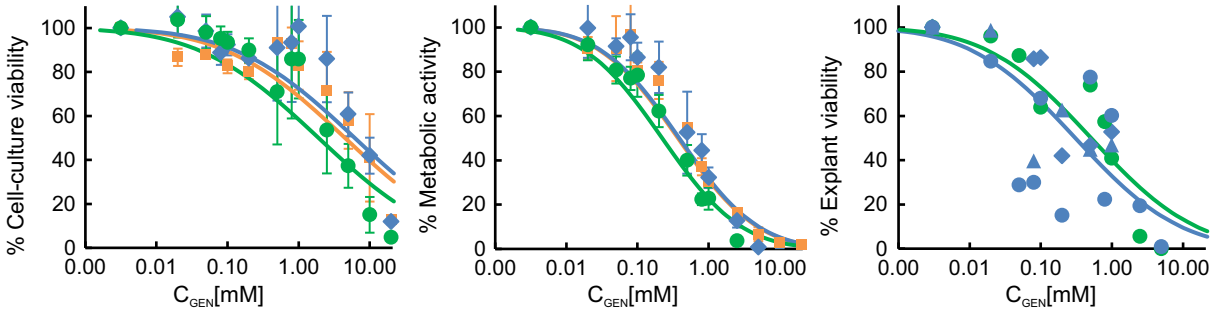

Fig. 3. (Left) Normalized cell viability measured by live/dead assay. Data points are averages from different animals ($n_r = 3$). Each measurement was performed with multiple technical replications ($n_m = 4$). The plotted standard deviations represent the variability between animals and not of technical replication. The probit model (goodness of fit: $p = 1$; common slope: $p = 0.219$) indicated significant effects of dose and time (dose: $p < 0.0001$; time: measured as a reduced axes intercept of the probit model for the 144 h group, meaning that the horizontal location of the curve is shifted to lower concentrations: $p < 0.05$). (Middle) Averaged ($n_r = 3$ and $n_m = 4$) normalized cell metabolic activity according to alamarBlue assay. The probit model (goodness of fit: $p = 1$; common slope: $p = 0.907$) indicated significant effects of dose (dose: $p < 0.0001$). There was no significant difference with time (axes intercept: $p > 0.05$). (Right) Normalized cell viability of explants from different horses as assessed by Trypan blue. The probit model (goodness of fit: $p = 1$; common slope: $p = 0.477$) indicated significant effects of dose (dose: $p = 0.03$). There was no significant difference with time (axes intercept: $p > 0.05$). Here, each data point is a measurement from a sample (averaged over duplicate measurements ($n_m = 2$) and this variability is not plotted). Note that, since $\log_{10}(0\ mM)$ is not defined, this data point was plotted at twice the distance between the two lowest non-zero concentrations on the $\log_{10}$ scale, therefore at 0.003 mM. Lines represent the probit models. ■ 24 h GEN treatment, ◆ 72 h GEN treatment, ● 144 h GEN treatment.

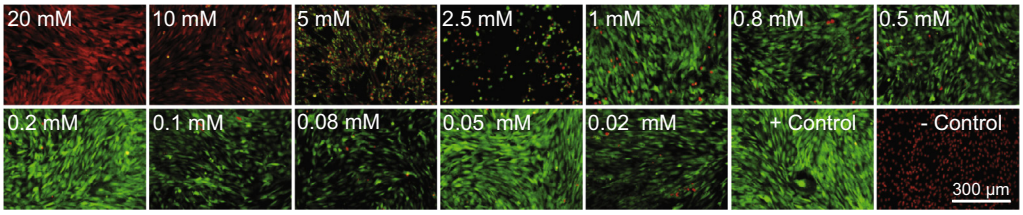

Fig. 4. Cell viability tested by live/dead assay. Red-stained cell nuclei indicate cell death. Green staining indicates vital cells. The present images were taken after 72 h of GEN treatment. Magnification:×10; C+: positive (sham) control; C−: negative control (ethanol-treated cells). Visible cell death is apparent at GEN concentrations starting at 2.5 mM, increasing in a dose-dependent manner. An elongated morphology is phenotypic of healthy tenocytes.

Table 2
Relative potency/toxicity (the ratio of the slope from the regression models) was used to estimate the effect of treatment time on cells.

Test	Comparison	Relative potency	LCI	UCI
Live/dead	24 h vs.72 h	0.73	0.22	2.24
	24 h vs. 144 h	2.35	0.78	7.74
	72 h vs. 144 h	**3.23**	**1.01**	**12.13**
alamarBlue	24 h vs.72 h	0.96	0.44	2.11
	24 h vs. 144 h	1.67	0.78	3.92
	72 h vs. 144 h	1.74	0.79	4.18
Trypan blue	72 h vs. 144 h	0.60	0.00	20.95

A significant effect is marked by a relative potency (between two treatment times) that is different than one (the number 1 indicates no change). This is true, if the upper 95% confidence interval (UCI) and lower 95% confidence interval (LCI) do not include one. Significant difference is noted bold.

concentrations (Fig. 7 and Table 4). During tensile mechanical testing, one triplet failed during mechanical preconditioning and was therefore excluded from further analysis. Tangent elastic modulus increased significantly, by 34%, after 20 mM GEN treatment, as assessed by the paired (Student's) *t*-test (Fig. 8 and Tables 4 and 5). MANOVA for blocked design (ANOVA for complete randomized block design was replaced due to violation of model assumptions) also showed a significant treatment effect on the elastic modulus. A significant 23% increase was observed in the 5 mM treatment group. A non-significant 14% increased elastic modulus was observed in the 1 mM group compared to controls using post hoc pairwise comparison. Relaxation, measured as relative stress decay over 300 s, was not significantly reduced due to crosslinking (0 mM: 10.3 ± 1.1%, 1 mM: 10.2 ± 1.6% and 5 mM: 9.5 ± 1.0%, $p = 0.231$). This measure of viscoelasticity was nevertheless larger in controls, and became smaller with increasing concentration. All further parameters assessed by two-way ANOVAs for the concentrations 1 and 5 mM, as well according to *t*-test on the 20 mM group, remained unaffected (Tables 4 and 5). Finally, treatments had no statistical effect on tissue swelling (6.0 ± 6.7%, $p = 0.948$) from an initial water content of 75.2 ± 4.8%. Also, initial lengths were very similar between groups (23.7 ± 1.2 mm, $p = 0.796$).

4. Discussion

Crosslinking of collagen-based implants has been widely used to augment their strength, elasticity, and resistance to fatigue-induced mechanical damage and premature degradation by the host system [5]. Crosslinking can improve implant survival within challenging mechanical environments, such as the cardiovascular and musculoskeletal systems. Genipin (GEN) is a plant-derived collagen crosslinking agent that has been demonstrated to be highly mechanically effective yet substantially less cytotoxic than traditional chemical crosslinking agents like glutaraldehyde. Given the efficacy and relatively low cytotoxicity of GEN, it has emerged as a candidate for in vivo application, with various studies demonstrating proof of principle for in situ biomechanical efficacy in treating keratoconus of the eye [38] and ruptures of the intervertebral disc annulus [8,39]. Our own work has shown it to be capable of arresting damage propagation in an in vitro model of tendon tear, being able to restore normal levels of tissue strains [5].

While GEN seems to offer promise as an in vivo collagen crosslinking agent, it has been reported to be cytotoxic at moderate concentrations, which vary depending on cell type (typically 0.5–5 mM). Until now it has been unclear whether a dense collagen matrix of connective tissues, like that in tendon, could be biomechanically augmented in situ without adverse consequences for the resident cell populations. The present study sought to define ranges of cytotoxicity for tendon cells, and then determine whether functionally relevant changes in tissue mechanics could be attained at these concentrations and exposure times. The overall goal was to better delineate the range of potential in situ applications that GEN may have, and provide dosing guidance for the development of delivery strategies.

Table 3
Selected potencies/toxicities of GEN treatment at 72 h: a probit regression model was used to estimate at which concentrations a change in tendon cell viability, metabolic activity or motility occurred (the relative effect).

Relative effect (% of control)	Cell culture viability (Live/dead)			Metabolic activity (alamarBlue)			Explants viability (Trypan blue)			Cell motility (scratch wound)		
	Estimated c_{GEN} (mM)	LCI (mM)	UCI (mM)	Estimated c_{GEN} (mM)	LCI (mM)	UCI (mM)	Estimated c_{GEN} (mM)	LCI (mM)	UCI (mM)	Estimated c_{GEN} (mM)	LCI (mM)	UCI (mM)
90	0.12	0.04	0.31	0.03	0.01	0.06	0.01	7.8E−10	0.51	0.62	0	18.47
80	0.5	0.2	1.1	0.07	0.03	0.1	0.03	1.2E−05	40.8	0.74	0	766
70	1.2	0.5	2.9	0.13	0.07	0.2	0.1	0.001	18307.8	0.9	0.002	1.0E+08
60	2.8	1.2	7.1	0.2	0.13	0.4	0.2	0.004	1.6E+07	1.1	0.05	3.1E+16
50	6.2	2.7	16.9	0.4	0.2	0.7	0.3	0.01	1.6E+10	1.3	0.17	2.2E+26
40	13.3	5.5	41.7	0.6	0.4	1.2	0.6	0.03	2.3E+13	1.6	0.31	1.8E+37
30	30.4	11.6	112.7	1.1	0.6	2.1	1.2	0.1	6.3E+16	2.0	0.5	3.3E+49
20	79.7	27.2	370.4	2.1	1.1	4.4	2.9	0.1	7.5E+20	2.6	0.7	7.5E+63
10	303.9	85.9	1986.9	5.1	2.6	12.4	9.3	0.3	3.8E+26	3.6	1.0	9.8E+81

The relative effects refer to sham-treated controls (0 mM GEN) as 100%: for example, at 6.2 mM GEN (c_{GEN}) 50% of cells survive compared to untreated controls. LCI: lower 95% confidence interval; UCI: upper 95% confidence intervals.

Table 4
Summary of statistical tests of the main experimental outcomes along with *p*-values.

Measures	Statistical tests	Repeated measure/block	c_{GEN}
Cell motility			
Migration speed	Two-way ANOVA	0.371	<0.0001
DSC			
Denaturation temperature	One-way ANOVA	–	**<0.0001**
Gene expression			
Casp 3 ($2^{-\Delta CT}$)	Friedman's test	–	0.882
Col A1 ($2^{-\Delta CT}$)	Friedman's test	–	**0.01**
MMP 1 ($2^{-\Delta CT}$)	Friedman's test	–	0.607
Mechanical test			
Relaxation	Two-way ANOVA	**0.01**	0.231
Elastic modulus	Two-way MANOVA	**<0.0001**	**0.011**
	t-test	–	**0.014**
Yield stress	Two-way ANOVA	**<0.0001**	0.395
	t-test	–	0.227
Yield strain	Two-way ANOVA	**<0.0001**	0.384
	t-test	–	0.156
Ultimate strength	Two-way ANOVA	**<0.0001**	0.387
	t-test	–	0.187
Strain at ult. strength	Two-way ANOVA	**0.01**	0.906
	t-test	–	0.397
Energy up to yield	Two-way ANOVA	**0.01**	0.638
	t-test	–	0.444

Statistical significance ($p < 0.05$) is noted by bold text. Measures: indicated are the tests used and the parameters that were analyzed. Statistical tests: Indicated are the tests used to assess the measures. Parametric tests were used when the model assumptions were met. Otherwise, as indicated, an appropriate non-parametric alternative was used. Repeated measure/block: *p*-values for all experiments are given. Some experiments were repeated multiple times and therefore the tissue/cells came from different horses. In the analysis a corresponding blocking factor was added to the ANOVAs. c_{Gen}: *p*-values given for the factor GEN concentration (from 0 to 20 mM). For the mechanical tests the 20 mM group was separately assessed with own-paired controls.

To provide a basis for effective in vivo dosing, we used probit regression to fit our experimental data. For instance, the model predicts that approximately 50% of cells would remain viable at GEN concentrations of 6.2 mM applied for 72 h (Table 3), with decreasing cell viability after more prolonged incubation (Fig. 3 and Table 2). While many cells remain alive at concentrations of 5 mM or less for the time spans we studied, effects on cell metabolism occurred at substantially lower concentrations. The probit model predicts a 50% drop in metabolic activity at a concentration of 0.4 mM after 72 h, with a trend to additional decreases in cell metabolism after 144 h of treatment (Fig. 3 and Tables 2 and 3). These findings were consistent with reduced cell motility at similar concentrations (Fig. 5 and Table 3). While reduced collagen I expression was also consistent with reduced metabolic activity, apoptosis markers and matrix degradation markers were not affected – a favorable finding regarding potential of GEN for in vivo application. Functional, physical effects on the matrix were statistically significant only at the tested concentration above 5 mM, and were independent of time of incubation (Figs. 6 and 8 and Table 5). We attribute these effects to homogeneous crosslink formation throughout the explants seen by fluorescence (Fig. 2). Interestingly, GEN crosslinking of cartilage scaffolds has an increasing effect on mechanical properties with longer treatment [16], and that effect can start from 2 h on, as measured in collagen gels [30]; similarly, GEN crosslinking increases the mechanical properties of rat tail tendon within 4.5 h [9]. Therefore, it is not clear whether our lack of time dependence is a result of underpowered experiments, low sensitivity of the tests or actual independence from the time of incubation.

Although a main aim of the work was to identify "cell-safe" and "matrix-effective" GEN dosing guidelines, it is clear that these objectives may be mutually exclusive to some extent. It appears that 5 mM (and maybe slightly lower concentrations) are able to induce relatively rapid crosslinking while leaving subpopulations of resident cells viable. Toward clinical application of in situ crosslinking to arrest tendon tear propagation, we believe a dosage of 5 mM or slightly lower with a dosing duration of 72 h would be a reasonable starting point for in vivo study. Although the degree of cytotoxicity that can be tolerated in vivo will vary according to the targeted tissue and clinical indication, properly balancing the need for rapid improvement in tissue function against the long-term consequence of altered cell and matrix metabolism will be imperative. No less important will be the need to develop effective vehicles for targeted crosslink delivery. Delivery of exogenous crosslinks that augment tissues at the intended site without adversely affecting neighboring tissues (e.g. tendon sheaths) represents a major challenge and is a thrust of ongoing work in our laboratory.

While robust augmentation of elasticity at 5 and 20 mM GEN concentrations is in line with our previous work at 20 mM [9], the mechanical effects in the present study were less drastic. This can be partly attributed to the slightly reduced sample number used here. Even considering the effects of sham incubation, which yielded trends of increased elastic modulus, ultimate strength and corresponding strain by 10–15% over the native tissue (data not shown), we remain confident of GEN augmentation effects in the present treatments.

When comparing the current studies to other investigations of GEN on tissues and cells, the present observed lack of time-dependent effects on cytotoxicity for culture periods of up to 72 h echoes previous studies on osteoblastic cells [24]. This study reported no observable cytotoxic effects in 0.044 mM GEN solution, while 0.44 mM GEN induced a 50% reduction in metabolic activity of fibroblasts [25]. These findings are in close agreement with our model prediction of 50% reduction in cell metabolism at 0.38 mM (95% confidence intervals: 0.22 and 0.69 mM). These results somewhat contradict other reports of limited cytotoxic effects on chondrocytes within explants treated up to 42 days at concentrations of 0.22 mM, and this while attaining mechanical enhancement of the tissue [16]. They also stand in contrast with another study reporting little cytotoxicity after crosslinking porcine heart valves in 8 mM GEN while obtaining a near doubling of stiffness [27].

While the current study was intended to be fairly comprehensive, several limitations must be noted. First, focused investigation

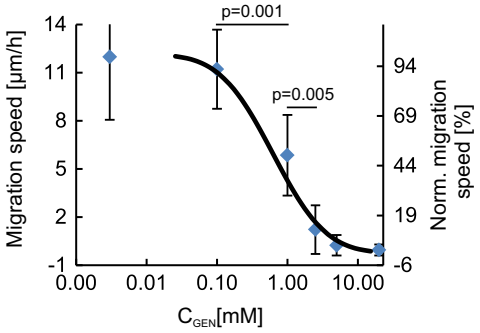

Fig. 5. Left axes: cell migration speed ($\mu m\ h^{-1}$) was averaged over time of image acquisition after scratch application ($n_m = 3$) and over experiments in different animals ($n_r = 3$). Horizontal bars indicate significant differences. Only significant differences between adjacent groups were plotted, as all other pairwise comparisons were significantly different. Right axes: probit fit of normalized cell migration speeds. 0 mM GEN is plotted at 0.003 mM. ◆ 72 h GEN treatment.

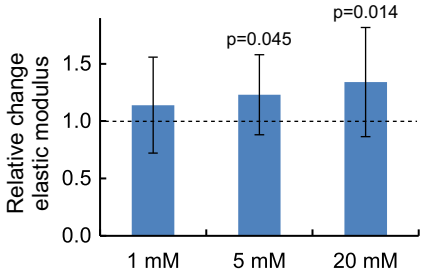

Fig. 8. Relative changes of elastic modulus compared to sham-treated controls (dashed line). The 20 mM group was separately assessed with its own matched controls, since the use of samples dissected into quadruplicates resulted in overly large inter-quadruple variability. This would have precluded the blocked experimental setup necessary to identify effects of crosslinking.

in narrower windows was deemed to be beyond the scope of this study. Such investigations will be necessary according to the clinical indication and the eventual GEN delivery approach that is utilized – aspects that were not explicitly addressed in the current study. Further, whether the effects we observed prove generally applicable to other dense connective tissues and clinical applications remains to be investigated. Nonetheless, the current study provides a useful baseline for dosage guidance in the design of future in vivo studies, and will be helpful in interpreting their outcome. As a further limitation, we observed slight increases in pH (maximally: 0.2) that were attributable to the addition of low GEN concentrations up to 2.5 mM. Since GEN crosslinking, self-polymerization before crosslinking and the crosslink effects on the physical properties of biopolymers have all been shown to be pH dependent [11,12], we cannot exclude confounding effects of these pH differences. However, the scales used to assess the effects of pH in those studies were at least tenfold the differences described here. Also, such small differences have only a marginal effect on cell viability and metabolism.

Finally, we note that the occasionally large variability within the various performed experiments can be attributed to similarly large variability across animals, and across specimens from a single donor tissue. Inter-specimen variability can be attributed to handling factors like anatomical sampling location, difficulties when cutting the strips along the main collagen direction and challenges in assessing the wet weight of small samples (hydration and dehydration effects). A further limitation is that the present study focused only on acute and relatively rapid crosslinking effects,

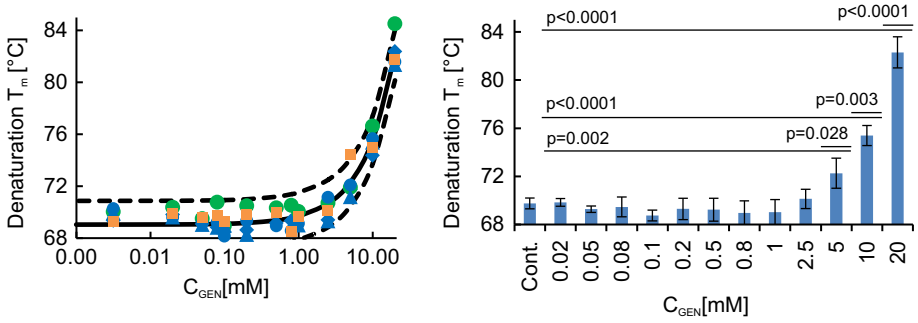

Fig. 6. (Left) denaturation temperatures as measured by DSC. Fit: linear model ($t_m = 0.66\ °C/mM \times [GEN]\ mM + 69.26\ °C$, $R^2 = 0.96$) with $\log_{10}$ transformed concentrations and 95% confidence intervals. 0 mM GEN is plotted at 0.003 mM. ■ 24 h GEN treatment, ◆ 72 h GEN treatment, ● 144 h GEN treatment. (Right) Bars indicate significant differences from post hoc pairwise comparison with a significant main effect. Only significant differences between adjacent groups or to controls were plotted as all other pairwise comparisons were significantly different, except below a concentration of 2.5 mM, for which there was no effect. Markers of the same color but different shape (■, ◆ and ●) indicate samples from the same experimental condition but from different horses.

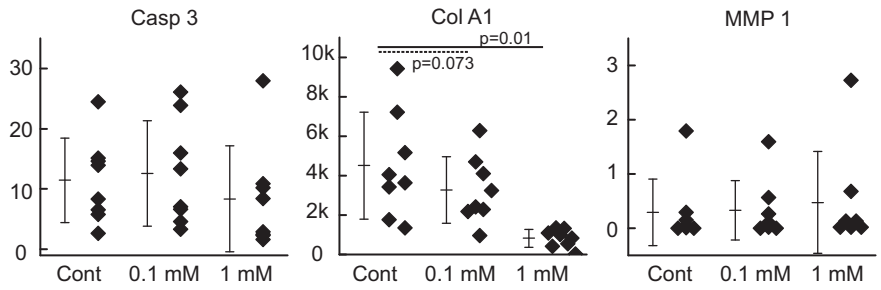

Fig. 7. Relative gene expression ($2^{-\Delta CT}$) normalized to an internal standard (housekeeping gene: GADHP): the non-parametric counterpart (Friedman's test) of a two-way blocked ANOVA was run with appropriated post hoc pairwise comparison since ANOVA assumptions were not met. A 5 mM GEN group was also tested; however, due to the crosslinking, insufficient RNA could be isolated and therefore the group was dropped. The dashed line indicates a statistical trend ($p \leqslant 0.1$).

Table 5
The main results from the mechanical tests.

		Treatment				
		0 mM[a]	1 mM[a]	5 mM[a]	20 mM[b]	0 mM[b]
Elastic modulus	(Mpa)	419 ± 145	436 ± 121	**483 ± 127[1]**	**434 ± 70[2]**	342 ± 75
Yield stress	(Mpa)	30 ± 13	32 ± 13	34 ± 12	25 ± 6	20 ± 7
Yield strain	(%L_0)	8 ± 2	8 ± 1	8 ± 1	7 ± 1	8 ± 2
Ultimate strength	(Mpa)	40 ± 18	43 ± 18	45 ± 19	28 ± 6	24 ± 7
Strain at ult. strength	(%L_0)	15 ± 4	15 ± 3	15 ± 4	10 ± 3	11 ± 2

Significant differences are noted in bold: (1) post hoc pairwise comparison to controls: $p = 0.045$; (2) paired t-test: $p = 0.014$.

[a] The concentrations 0, 1 and 5 mM were tested in blocked fashion, where for each concentration one tendon strip from the same triplet was assigned. A total of 13 triplets ($n_r = 13$) were used and analyzed by two-way ANOVA for a complete randomized block design.

[b] The concentration 20 mM was assessed in separate experiments, where tendon strips were cut in pairs ($n_r = 11$), subjected to either 20 or 0 mM and analyzed with a paired t-test. These paired-testing experiments used slightly different incubation protocols (buffer instead of medium) and a different cross-sectional area measurement techniques, which might explain the overall lower values compared to (a).

neither investigating whether long-term administration of GEN at lower doses (1 mM or less) could possibly achieve a cumulative functional effect with less pronounced cytotoxicity nor observing the continuity of the documented effects. Finally, on a more basic level, we did not investigate how GEN crosslinks are actually formed or remain stabilized within tissue – factors that may vary depending upon the dosing concentration and time. While this information may be helpful to interpreting our results, we note that others have reported that the stabilizing effects of GEN are comparable to glutaraldehyde [40].

5. Conclusions

The crosslinking agent genipin is increasingly invoked for the mechanical augmentation of collagen tissues and implants, and has been demonstrated to arrest mechanically driven tissue degeneration. 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 for 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 to predict the corresponding degree of cell loss and diminished metabolic activity that can be expected. The data indicate that rapid mechanical augmentation of tissue properties can only be achieved by accepting some degree of cytotoxicity, but that post-treatment cell survival may be adequate to eventually repopulate and stabilize the tissue. On this basis, it can be concluded that development of delivery strategies and subsequent in vivo study is warranted.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

The authors gratefully acknowledge the contribution of Remo Friedrich in developing and validating the cross-sectional area laser scanner and of Domenica Brütsch for performing parts of the mechanical testing. The authors also would like to thank Yufei Li and Hansrudolf Sommer for their expertise and contribution to the mechanical testing. The support of Prof. Raffaele Mezzenga and Johannes Haberl from the Laboratory of Food and Soft Materials of ETH Zurich for access and assistance to the differential scanning calorimeter is further gratefully acknowledged. This work was funded by Swiss National Science Foundation grant number 205321–118036.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.12.048.

Appendix B. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 2–6 and 8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2013.12.048.

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