

EFFECT OF A NATURAL CROSSLINKING AGENT (GENIPIN) ON TENDON LONGITUDINAL AND TRANSVERSE TENSILE PROPERTIES

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INTRODUCTION

Biological scaffolds have been utilized in the repair of connective tissue defects, construction of vascular grafts, and drug delivery systems. The successful function of these constructs requires mechanical properties comparable to normal tissue, timely integration during healing, biocompatibility, and functional recovery at the site of insertion [1]. Aldehyde-fixed bioprostheses are a common form of bio-scaffold that are characterized by the formation of crossbridges between free amino side chains. While these implants feature sufficient mechanical strength and low antigenicity, their use is complicated by their high cytotoxicity [1,2]. Genipin is a naturally occurring crosslinking agent that has demonstrated similar utility to aldehyde-fixation with significantly lower toxic effects. Its optimal activity occurs in conditions similar to those in living tissue and makes this substance a potential candidate for use in the development of bio-scaffolds [3,4].

Early studies have demonstrated the successful use of genipin in pericardial tissue and in the creation of a drug delivery system [5,6]. The objective of this study was to quantify the elastic tensile properties of tendon fixed in a genipin solution to determine if genipin may serve a role in the development of connective tissue implants.

MATERIALS AND METHODS

Specimen Preparation

Four sheep hind limb flexor tendons were used in this study. Following harvesting, flexor tendons were divided into 20 mm long sections and microtomed to achieve a uniform thickness. Parallel samples aligned in either the longitudinal or transverse tendon direction were prepared using a custom-made razor die. Sections from each flexor tendon were placed in baths of either phosphate-buffered saline (PBS) or 0.5% genipin in PBS and allowed to soak at room temperature for either 12, 24, or 72 hours. A 0.1% sodium azide was included in all baths to inhibit bacterial growth.

Tensile Testing

All testing was performed on an Instron 5543 mechanical testing apparatus. The width and thickness of each sample was determined optically under magnification. A pair of marker lines parallel to the sample width were imprinted with Verhoeff's tissue stain for use in optical strain analysis. Both ends of the sample were glued into water-resistant sandpaper, gripped, and loaded into the Instron. Tensile testing was performed at room temperature in a PBS bath. Transverse samples were preloaded to 0.005 N and held at a constant extension for 600 s. At the conclusion of this time the sections were stretched at a strain rate of 1%/s until failure occurred. Longitudinal samples were preloaded to 0.5 N, pre-conditioned for 15 cycles between 0% and 3% strain, and held at a constant extension for 600 s. Samples were then stretched at a strain rate of 1%/s until failure. Images were recorded at one-second intervals during the stretch-to-failure stage of the testing protocol. Custom-written optical strain analysis software was used with the recorded images to determine the change in tissue strain during tensile extension [7]. Stress was calculated by dividing the force by the initial area. Modulus was calculated using a linear regression of the stress-strain response within the linear region of the curve. The effect of treatment (genipin vs. control) and duration of treatment (12, 24, and 72 hours) on stiffness, modulus, and cross-sectional area was determined using a two-factor analysis of variance, with significance $p < 0.05$.

RESULTS

The stiffness and modulus of samples that were aligned in the transverse direction (i.e., perpendicular to the collagen fiber populations) were significantly larger following genipin treatment than in control (Fig 1 and 2). Stiffness and modulus were also significantly affected by time of treatment. For example, the transverse tendon stiffness increased by a factor of 1.5X at 12 hours, and the modulus increased nearly 1X compared to control values. This effect was even more dramatic at 24 and 72 hours of genipin treatment. There was no significant difference in the cross-sectional areas of samples soaked in genipin compared to controls (mean area $4.5 \pm 0.7 \text{ mm}^2$).

For samples aligned in the longitudinal direction (parallel to the collagen fiber population) the modulus and cross-sectional area were significantly increased by genipin treatment. The increase in stiffness was nearly significant ($p=0.075$). The genipin-treated modulus and stiffness were 3-4X greater than in the control tendons at 12 and 24 hours. For example, the average modulus in control specimens soaked for 12 h in PBS was 32 MPa while the modulus in specimens fixed in genipin increased to 202 MPa (Fig 3). The cross-sectional area was 30% lower in genipin-treated samples compared to controls. Time of fixation was not shown to significantly affect the mechanical properties or cross-sectional area between specimen groups.

Figure 1. Stiffness (N/mm) for transverse aligned tendon following PBS (control) and genipin treatment

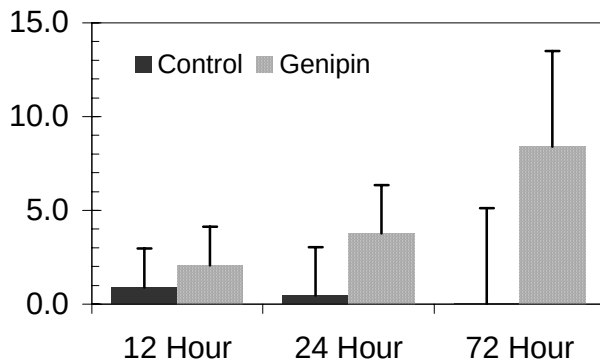
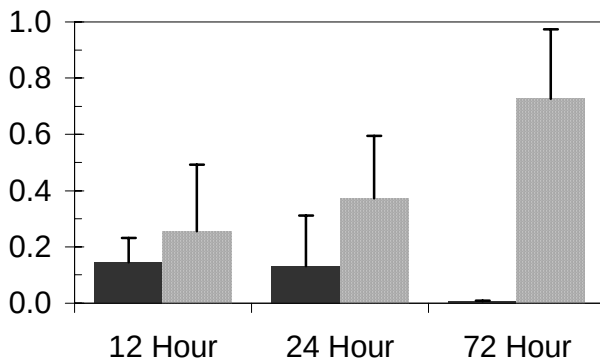


Figure 2. Modulus (MPa) for transverse aligned tendon



DISCUSSION

This study demonstrated that genipin treatment increases both the stiffness and modulus of tendon after 12 to 72 hours of soaking at room temperature. Previous studies have utilized genipin in pericardial and drug delivery applications [5,6], however, this is the first study to examine its potential use in bio-scaffolds for orthopaedic applications. These preliminary results suggest that this crosslinking agent has potential utility in soft tissue repair and tissue-engineering applications.

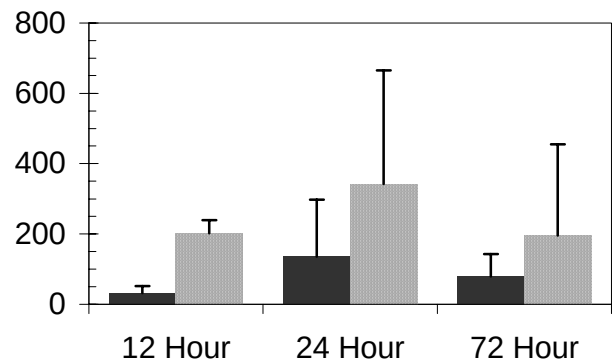
Genipin is a naturally occurring crosslinking agent obtained from geniposide, a substance isolated from the flowering plant *Gardenia jamaicensis* Ellis [8]. Genipin causes crosslinking of free amino groups, including lysine, hydroxylysine, and arginine and genipin forms intramolecular and intermolecular crosslinks within collagen [3,8]. It has been shown to be over 5000X less cytotoxic than other crosslinking agents, including glutaraldehyde [3]. In addition, the optimal conditions for activity are pH 7.4-8.5 and temperatures 25-45

C [4], which are reasonable ranges for working with tendon and other orthopaedic tissues.

In the transversely-oriented samples, we observed material property degradation of the control samples after 24 and 72 hours of soaking in PBS when compared to 12 hours of PBS soaking. This occurred even in the presence of a bacterial growth inhibitor. Future untreated control studies will be performed without PBS soaking in order to determine whether any degradation occurred during the 12 hour treatment. Since sodium azide was included in all of the soaking preparations, it is likely that the decrease in mechanical properties was due to glycosaminoglycan loss. This mechanism is also supported by the occurrence of significant degradation in only transverse samples and not fiber-aligned samples. Thus, we expect that the function of bio-scaffolds, which would be oriented parallel to connective tissue fibers, would not be adversely affected by degradation; however, additional studies are needed.

In summary, these findings are promising for the application of genipin to the development of a potential bio-scaffold for tendon and other orthopaedic soft tissues such as ligament, meniscus, cartilage, and intervertebral disc. Genipin has an advantage over other cross-linking agents in that it has comparable fixing ability but lower cytotoxicity. Future studies will optimize the fixing parameters required for successful bio-scaffold implementation. In addition, *in vivo* studies will examine genipin-fixed tendon biocompatibility.

Figure 3. Modulus (MPa) for fiber-aligned tendon



REFERENCES

1. Bailey, A.J., 2000, Wound Repair Regen, 8, pp 5-12.
2. Jayakrishnan, A., and Jameela, S.R., 1996, 17, pp471-484.
3. Sung, H.W., Liang, I.L., Chen, C.N., Huang, R.N., and Liang, H.F., 2001, J Biomed Mater Res, 55, pp 538-546.
4. Sung, H.W., Chang, Y., Liang, I.L., Chang, W.H., and Chen, Y.C., 2000, J Biomed Mater Res, 52, pp 77-87.
5. Sung, H.W., Chang, Y., Chiu, C.T., Chen, C.N., and Liang, H.C., 1999, J Biomed Mater Res, 47, pp 116-126.
6. Mi, F.L., Tan, Y.C., Liang, H.F., and Sung, H.W., 2002, Biomaterials, 23, pp 181-191.
7. Derwin, K.A., Soslowsky, L.J., Green, W.D.K., and Elder, S.H., 1994, J Biomechanics, 27(10), pp 1277-1285.
8. Sung et al, 1998, J Biomed Mater Res, 42, pp560-7

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