Mechanical Properties of Mineralized Collagen Type I Rat-Tail Tendon Fascicles

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ABSTRACT

Type I collagen fibers transmit forces, dissipate energy, and prevent mechanical failure in normal tissues. Mineralization of collagen fibers is proposed to strengthen these structural proteins as a possible therapeutic option for small tears in soft tissues such as cemental tears in periodontal ligaments. In the present study, collagen fascicles extracted from the tail tendons of male Sprague-Dawley rats were tested in a MiniMat tensile tester at different strain rates. The fascicles were previously immersed in SBF to induce mineralization and were maintained in a moist condition during mechanical testing. Mineralization of the fascicles resulted in an increase in the modulus of elasticity and tensile strength with minimal change in percentage elongation to failure. From SEM/EDS, calcium phosphate deposits formed on the fascicles indicating a high probability of intrafibrillar mineralization resulting in the observed improvement in mechanical properties.

Keywords: Collagen fascicles, calcium phosphate, mechanical properties, rat tail tendon.

I. INTRODUCTION

Collagen is the most important structural protein and serves to support and transfer loads in the human body. Load-bearing elements such as muscles, tendons and ligaments consist of collagen fascicles, which are in turn composed of collagen fibrils and interspersed with cells and non-collagenous matrix including proteoglycans. The mechanical properties of the hierarchical structures of collagen have been studied quite well [1]-[4]. Collagen is also the main organic component and the structural unit of bone. In this hard tissue, collagen is arranged in the form of lamellae interspersed with calcium phosphate (hydroxyapatite) nanocrystals embedded in collagen fibrils. The mineralization process, wherein hydroxyapatite crystals are incorporated in the collagen structure increases the mechanical properties of bone tissue such as elastic modulus and ultimate tensile strength while lowering the fracture strain of the fibers. These mineral aggregates increase the storage of elastic energy and energy dissipation in the structure affecting fracture and injury processes. Since mineralized fibrils comprise the elementary unit of the complex bone structure, it is highly important to understand the manner in which the degree of mineralization affects the structure and properties of the fibrils and possibly the mechanism involving the mineralization of collagen fibrils [5].

Earlier studies indicate that the calcium phosphate crystals embed in the D-band gaps [6] of the collagen fibrils, resulting in an increase in the tensile strength while decreasing the elasticity of the fibers. Computational studies suggest that in unmineralized collagen fascicles, the gaps in the D-bands are used as sacrificial bonds which increase the length of the fascicles as an energy dissipation mechanism, in cases where the tensile strength is exceeded [7]. The location of the hydroxyapatite nanocrystals in the D-band gaps is bound to affect the mechanical behavior of mineralized collagen fibers by acting as a deterrent to the sliding of the staggered fibers. It has been reported that as the mineral density of bone increases, the corresponding mechanical properties also increase above and beyond those of pure collagen fibrils [8].

A mechanical model with a staggered mineral particle arrangement has been proposed to account for the increase in fracture stress of mineralized collagen fibrils [9]. Another study on avian tendons demonstrated that mineralization increased their tensile strength and modulus [10]. This increase in mechanical properties may be useful in possibly prescribing therapies to treat and repair minor damage in muscles, tendons and ligaments. Cemental tears in periodontal ligaments although not frequent in occurrence, do require treatment and understanding the manner in which mineralization is accomplished in soft tissues might lead to viable therapeutic options in the future [11]-[13]. Lesions in these and other connective tissues which occur due to trauma and aging, usually result from tears in the collagen fibrils that compose these connective tissues. Physical therapies, including electrotherapy and ultrasound treatment (these are passive modalities which may be ineffective), are the current options to heal these tears and in the more severe cases, surgery becomes the only reparative option. Mineralization of these damaged structures may possibly be an important nonsurgical alternative to strengthen or heal injury to connective tissues. Nevertheless, not much information is available in literature regarding the mechanical behavior of mineralized soft tissues. In lieu of the dearth of experimental
data about the behavior of mineralized soft tissues, further studies must be performed to understand the mechanical response of mineralized collagen structures to applied loads.

In this paper, the mechanical properties of collagen fascicles, extracted from rat tail tendons and subjected to varying degrees of mineralization, are determined using tensile testing at various strain rates. These will be compared with control collagen fascicles without mineralization. The study is expected to provide information which may have much utility in the prescription of effective nonsurgical treatments for strengthening weak or injured soft connective tissue in the body.

II. MATERIALS AND METHODS

A. Extraction of Collagen Fascicles

Frozen Sprague-Dawley rat tails measuring about 13 cm in length each were purchased from Bioreclamation, NY and stored at -20 °C. The rat tail tendons were later thawed by immersion in water at room temperature for five minutes. Each tail was sectioned transversely one centimeter from the root and two centimeters from the tip to remove attachments to the internal structure. From the distal end (tip) the skin was spliced two centimeters longitudinally and the four tendons in the tail were exposed by peeling back the skin. Care was taken to avoid any damage to the tendons. A sharp scalpel was utilized to sever the tendon before the vertebral connection after which the loose end of the tendon was firmly pulled to extract various fascicles. This same procedure was applied to all four tendons and as many fascicles as possible were extracted. The same process was adopted to harvest as many fascicles as possible from various tails. The extracted fascicles were immersed immediately in Phosphate Buffered Saline (PBS) solution.

B. Mineralization of Collagen Fascicles by Immersion in SBF

Some of the collagen fascicles were immersed in a Simulated Body Fluid (SBF) solution, which was prepared using 44.67 g/L of NaOH, 12.76 g/L of calcium acetate Ca(CH$_3$COO)$_2$; and 6.3 g/L of calcium dihydrogen phosphate Ca(H$_2$PO$_4$)$_2$·H$_2$O. NaOH was added finally to set solution pH at 11 [14]. SBF emulates the concentration of calcium and phosphate found in blood plasma. The immersion process is purely passive as the fascicles are left in a Petri dish for the proposed time intervals of 5 hours and 24 hours per experimental design prior to mechanical testing. The varying periods of immersion were carried out with the expectation of varying degrees of mineralization of the fascicles.

C. Scanning Electron Microscopy

Some fascicles immersed in SBF were subjected to critical point drying and sputter-coated with gold for observation in a JEOL scanning electron microscope. Images of the deposits on the surface of the fascicles were obtained. EDS semi-quantitative compositional data of the mineral particles which were embedded in the collagen fascicles were also obtained.

D. Mechanical Characterization

Tensile testing of the fascicles was carried out after the period of immersion in SBF per experimental design. For comparison, fascicles which were immersed for similar periods in PBS solution were also tested as control samples. The fascicles were subjected to tensile testing at three strain rates (2.5, 5 and 10 mm/min) after immersion in SBF for 5 hours and 24 hours respectively. The strain rates were chosen to mimic possible physiological activity of the collagen fibers during strenuous physical activity. Tensile testing was performed using a MiniMat Tensile Tester with a 20 N load cell. Special plastic inserts were used to grip the fascicles and prevent them from slipping during the test. The fascicles were hydrated with PBS solution during the test to prevent them from drying. Mechanical properties were determined from the resulting stress-strain diagrams.

III. RESULTS AND DISCUSSION

The results from the mechanical testing of the collagen fascicles are summarized in Table I. Fascicles immersed in PBS solution and tested at a strain rate of 2.5 mm/min showed an average Elastic Modulus of 13.5 MPa, while the average tensile strength for these samples was 0.63 MPa with a corresponding average percentage elongation to failure of 6.86%. An increase in the strain rate during tensile testing to 10 mm/min resulted in a slight increase in the average Elastic Modulus of the fascicles to 14.8 MPa, average tensile strength value of 1.14 MPa and an average elongation to failure of 9.44%. These values are similar to those reported in literature. Contrasting effects regarding the water content in soft tissues including fascicles on their mechanical properties have been reported [15]-[17], although there appears to be no effect on the mechanical behavior resulting from the presence of salts in the PBS solution [18]. The purpose of immersion of the fascicles in PBS after retrieving them from the rat tail tendons was only to maintain a constant water content in these tissues. In the case of these fascicles immersed in PBS, tensile testing at different strain rate does not appear to have an effect on the mechanical properties.

| TABLE I: MECHANICAL PROPERTIES OF COLLAGEN FASCICLES TESTED IN PBS AND SBF (N=10) |
|---------------------------------|----------------|----------------|----------------|
| Medium | Strain Rate [mm/min] | Elastic Modulus [MPa] | Tensile Strength [MPa] | % Strain at Failure |
|--------|----------------------|----------------------|----------------------|---------------------|
| PBS    | 2.5                  | 13.50 ± 2.50         | 0.63 ± 0.22          | 6.86 ± 2.35         |
|        | 5                   | 25.75 ± 6.61         | 1.72 ± 0.55          | 6.14 ± 1.53         |
| SBF    | 5                   | 20.12 ± 2.97         | 1.31 ± 0.19          | 6.99 ± 1.46         |
| 5 Hrs  | 10                  | 24.66 ± 5.50         | 1.55 ± 0.44          | 6.99 ± 1.46         |
| SBF    | 2.5                  | 28.70 ± 6.19         | 1.67 ± 0.53          | 6.12 ± 1.29         |
| 24 Hrs | 5                   | 27.64 ± 6.93         | 1.42 ± 0.31          | 6.56 ± 1.52         |
|        | 10                  | 25.72 ± 5.67         | 1.61 ± 0.69          | 6.63 ± 1.77         |

After controlled immersion in SBF, the average values of the mechanical properties for an immersion time of 5 hours yielded an average Elastic Modulus of 25.75 MPa, an average tensile strength of 1.72 MPa, and an average elongation to failure of 6.14% when tested at a strain rate of 2.5 mm/min. Increasing the strain rate to 5 mm/min resulted in an average Elastic Modulus of 20.12 MPa, an average tensile strength at 1.42 MPa, and the average elongation to failure at 6.56%. A further increase in strain rate to 10 mm/min yielded average
values of 25.72 MPa, 1.61 MPa, and 6.63% for Elastic Modulus, tensile strength, and elongation to failure respectively. Fascicles immersed in SBF for 24 hours exhibited mechanical properties which were similar to those for the 5-hour immersion in SBF regardless of the applied test strain rate. While there is a clear improvement in the mechanical properties of the rat tail tendon fascicles after immersion in SBF compared to those immersed only in PBS solution, a two-sample t-Test (equal or unequal variance assuming a normal distribution) suggested that there is no significant difference between the mechanical properties of the fascicles subjected to a 5-hour immersion and a 24-hour immersion in SBF, even when tested at three different strain rates.

It is important to highlight that some of the fascicles failed at the vicinity of the grips during testing but some others in different regions of the sample length. Similar behavior has been observed in other studies and have been attributed to stress concentration in the fascicle samples in the grip area or damage even prior to testing during the placement of the fascicles within the grips [2], [19], [20]. Also, the structure of the fascicles is expected to differ from sample to sample based on the nature of the microfibrils which compose each fascicle. This will also result in the sample failure occurring at different points of the sample length during mechanical testing. Additionally, there is definite variability in the mechanical properties of the fascicles even when sourced from the same rat tail sample from which the fascicles were extracted despite performing the tensile tests under the same conditions. Clearly, there exists a large degree of heterogeneity among the fascicles even from the same source. Besides, if differences in the rat tails based on the age and sex (in this study the tails were harvested from male rats) are factored in, one should expect a sizable variation in the mechanical properties. Another factor with possibly a lesser effect is sample dryness due to evaporation which is prone to occur the longer the testing time (slower strain rates result in samples with relatively lower humidity). Nevertheless, despite the variation in the experimental values, it is clear from the data presented in Table 1 that there is a definite increase in the Elastic Modulus and tensile strength values for the fascicles mineralized in SBF compared to those immersed solely in PBS.

Collagen has been generally acknowledged to act as a template in mineralized tissue [21], [22]. Although a clear explanation is yet to be elucidated in literature, it is believed that nanoscale components of bone mineral are incorporated into collagen at intrafibrillar sites. A previous study has reported the infiltration of hydroxyapatite through capillary action in the presence of poly-aspartic acid (pAsp) resulting in intrafibrillar deposition on collagen, while an absence of pAsp apparently led only to extrafibrillar precipitation of hydroxyapatite on collagen [23]. Current knowledge is unable to pinpoint the exact localization of the hydroxyapatite crystals in intrafibrillar collagen, although channels in the α-bands and e-bands have been suggested as probable positions [24]. Neither is there a plethora of data available regarding the possible control and incorporation of this mineral phase in intrafibrillar collagen. A recent study emphasizes that the incorporation of minerals in the intrafibrillar regions of collagen fibrils is not dependent on the ionic nature of the electrolyte which is used in the infiltration process but rather on the osmotic equilibrium of the mineral elements in the fluid in which the fascicles are immersed [25]. In light of this paucity in the knowledge of the location of minerals in collagen fibers, it is difficult to determine from the data obtained in the current study if calcium phosphate mineralization indeed occurred at intrafibrillar collagen sites during the immersion of collagen fascicles in SBF prior to mechanical testing. However, it is clear from the EDS data in Fig 1 that mineralization did take place, evidenced by the presence of elemental calcium and phosphorus in the fascicles. An SEM image in Fig 2 of a collagen fascicle immersed only in PBS indicates the absence of any deposit on the fascicles as is to be expected. Fig 3, on the contrary, is a SEM image of a fascicle which was immersed in SBF for 24 hours and shows clear indication of the formation of mineral deposits which can be deciphered to contain calcium phosphate when analyzed in conjunction with the EDS data. Similar deposition of hydroxyapatite particles on collagen fibrils through self-assembly has been reported earlier [26].

Based on observations in literature, it is expected that mineralized collagen will be stiffer and stronger than corresponding collagen structures which lack the presence of mineralization. In the present study, there is no denying the clear increase in the Elastic Modulus and tensile strength of mineralized collagen fascicles after immersion in SBF compared to unmineralized fascicles. In an earlier study, a four-fold increase in the stiffness of collagen scaffolds subjected to mineralization has been reported [8].

It is assumed that on the mechanism proposed in an earlier study [25] using computer modeling and high-resolution transmission electron microscopy, intrafibrillar mineralization of the fibers probably occurs in the fascicles immersed in SBF in this study. Furthermore, the calcium phosphate nodules that are visible in Fig. 3 and at a higher magnification in Fig 4 is in most probability, an indication of the saturation of the intrafibrillar collagen sites through mineralization and consequently resulting in the deposition of these mineral nodules in an extracellular fashion on the fascicles. Heavy intrafibrillar mineralization of collagen fibrils have been reported after these were immersed in a CaP mineralizing solution for 48 hours [25]. In the present study, it is quite probable that saturation of intrafibrillar sites in the collagen fibers by calcium phosphate minerals occurred even after a few hours of immersion in SBF. Hence the lack of a significant difference in the mechanical properties as a function of immersion time in SBF.

The results from the present study clearly indicate that mineralization of the collagen fascicles results in a significant improvement in their mechanical properties and hence point toward possible therapies for strengthening connective tissues through the process of mineralization of these tissues via administration of the mineral in situ. Furthermore, greater understanding of the mechanism of the mineralization of collagen fibrils and the corresponding change in properties may pave the way for advanced therapeutic methods to treat injuries to connective tissue. An additional benefit is the possibility of adopting different tissue engineering strategies to create viable collagen templates with targeted mineralization sites to successfully fabricate bone tissue [26], [27].
of the collagen fascicles immersed in SBF showing the presence of calcium (Ca) and phosphorus (P).

Fig 2. SEM image of an unmineralized collagen fascicle stored in PBS solution marked by the clear absence of mineral deposits.

Fig 3. SEM image of a rat tail tendon fascicle after immersion in SBF for 24 hours indicating particular deposits which appear to be extrafibrillar. Based on the EDS data in Fig 1, these deposits are most probably calcium phosphate.

Fig 4. High magnification image of the mineral deposits on the fascicles reveal binding to non-specific sites on the fibrils that comprise the fascicle. Lower hierarchical structures reveal banding typical of collagen fibers (red arrows).

IV. CONCLUSIONS

The results of this study indicate that the mechanical properties of collagen fascicles sourced from rat tail tendons are increased when mineralized in Simulated Body Fluid (SBF). SEM images and EDS data indicate that simple immersion of collagen fascicles in SBF results in calcium phosphate deposits that appear to be extrafibrillar. However, these can be an indication of mineral saturation as a result of intrafibrillar mineralization as construed from an earlier report in literature. This is also implied in the observation that there is no effect of strain rate on the mechanical properties after immersion of the fascicles in SBF for 5-hour and 24-hour durations. It is possible that saturation in the intrafibrillar mineralization is achieved after 5 hours of immersion of the fascicles in SBF. More detailed studies are clearly necessary to understand the mechanism of mineralization of collagen fascicles and effective means to accomplish this process for possible strengthening of collagen fibers in medical treatment of small tears or lesions. This process may serve as a nonsurgical treatment for the strengthening of soft tissues.

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CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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