ABSTRACT

The interplay between diabetes mellitus and the structural integrity of collagen has significant implications for tissue functionality and disease progression. The aim of this study was to empirically investigate the effects of ribose-induced glycation on the biomechanical properties of collagen fibrils, using atomic force microscopy for precise measurements.

Methodology. We used collagen fibrils from the common digital extensor (CDE) and superficial digital flexor (SDF) tendons of an adult bovine model to mimic the glycation processes that occur in diabetic pathology. The samples underwent controlled glycation by incubation with ribose for 24 hours and 14 days compared to phosphate buffered saline treated controls. A Bioscope Catalyst atomic force microscope (Bruker, USA) was used for all atomic force microscopy imaging in this study.

Scientific novelty. Our results show a marked increase in the elastic modulus of collagen fibrils after ribose treatment, indicating stiffening with glycation. Notably, SDF fibrils showed a greater increase in stiffness after 24 hours of ribose exposure compared to CDE fibrils, suggesting variations in glycation rates relative to fibril anatomy. Statistical analyses confirmed the significance of these findings and provided a model for understanding similar processes in human diabetes.

Conclusions. The different response to glycation observed between CDE and SDF fibrils prompts further investigation into the role of anatomical and structural factors in glycation susceptibility. Identification of tissues at higher risk of ribose-induced damage could lead to the development of targeted prevention strategies for diabetic complications. In addition, the potential for pharmacological intervention to inhibit glycation processes or enhance advanced glycation end products (AGES) degradation offers a promising avenue for mitigating the progression of diabetes-related complications. The results of this study highlight the potential of ribose-induced changes in collagen as a model for diabetes-related tissue changes and propose a mechanistic framework that could guide the development of interventions aimed at mitigating the effects of collagen-related diabetic complications.

Key words: glycation, ribose, common digital extensor (CDE), superficial digital flexor (SDF), tendons, atomic force microscope

АНОТАЦІЯ

Взаємодія між цукровим діабетом і структурною цілісністю колагену має значний вплив на функціональність тканин і прогресування захворювання. Мета цього дослідження полягала в емпіричному дослідженні впливу глікації, індукованої рибозою, на біомеханічні властивості колагенових фібрил, використовуючи атомно-силову мікроскопію.

Методологія. Ми використовували колагенові фібрили із сухожиль загального розгинача пальців (CDE) і поверхневого згинача пальців (SDF) моделі великої рогатої худоби, щоб імітувати процеси глікації, які відбуваються при діабетичній патології. Зразки піддавали контролюваній глюкозі через інкубацію з рибозою протягом 24 годин та 14 днів порівняно з контролем (сухожили, оброблені фосфатним буферним розчином). Атомно-силовий мікроскоп Bioscope Catalyst (Bruker, США) використовували для отримання зображень у цьому дослідженні.

Наукова новизна. Наші результати показують помітне збільшення модулю пружності колагенових фібрил після обробки рибозою, що вказує на усільнення навислодок глікації. Зокрема, фібрили SDF показали більше збільшення жорсткості після 24 годин впливу рибози порівняно з фібрилами CDE, що свідчить про
Introduction

Diabetes mellitus (DM) is a multifaceted chronic disease characterised by high blood glucose levels resulting from defects in insulin production, insulin action or both. It has become a global health crisis with a wide range of complications that reduce quality of life and increase the risk of mortality (Banday et al., 2020). A critical aspect of these complications is the non-enzymatic glycation of proteins – a process that is accelerated in the hyperglycaemic conditions prevalent in diabetes (Khalid et al., 2022). Glycation involves the attachment of sugar molecules to proteins, particularly collagen, the most abundant protein in the human body, resulting in the formation of advanced glycation end products (AGEs) (Twarda-Clapa et al., 2002). These AGEs have a profound effect on the structural and functional integrity of proteins, leading to altered tissue mechanics and contributing significantly to the pathogenesis of diabetic complications such as nephropathy, retinopathy and impaired wound healing (Singh et al., 2014). Tendon tears and ruptures are common and can be caused by trauma, degenerative disease and overuse (Breidenbach et al., 2014; No et al., 2020). Tendon tears and ruptures in people with diabetes may be related to hyperglycaemia-induced changes in collagen structure, as excess blood glucose reacts with collagen to form AGEs, making tendons stiffer and more susceptible to injury (Nichols et al., 2020). Diabetes is also associated with increased skeletal fragility and an increased likelihood of fractures (Kanazawa, 2017).

Although diabetes can be associated with increased bone mineral density, it is associated with compromised bone architecture and altered mineral properties (Cortet et al., 2019). The glycation process also interferes with the normal healing response, resulting in weaker repair tissue (Van Putte et al., 2016). In general, the accumulation of overly glycosylated collagens and the reduction in the levels of sulphated proteoglycans lead to morphological changes, including thickening of the basement membrane and increased thickness and hardening of the skin (Sternberg et al., 1985). In addition, diabetes-associated microvascular complications further compromise tendon health by restricting blood flow, which is essential for tissue maintenance and repair (Vithian & Hurel, 2010).

While there is extensive data on the glycation of extracellular matrix proteins, particularly collagens, in the context of diabetes, the specific impact of glucose modification on the mechanical structure and integrity of collagen, its interaction with cells and collagen turnover during disease progression remains poorly understood (Snedeker & Gautieri, 2014; Onursal et al., 2021). There is compelling evidence that collagen glycation plays a critical role in tissue fibrosis associated with diabetes (Snedeker & Gautieri, 2014). Collagen cross-linking in the body occurs via both enzymatic and non-enzymatic pathways, the latter leading to the formation of advanced glycation end products such as pentosidine and glucosepane (Saito & Marumo, 2015). Investigations into the molecular mechanisms underlying the disruption of collagen structure and metabolism in diabetes have highlighted the important role of AGEs in these changes (Picke et al., 2019; Zgutka et al., 2023).

Collagen, a key component of the extracellular matrix, is integral to the structural framework of tissues throughout the body, providing mechanical stability and elasticity. More generally, fibrous polymers are the major...
building blocks of all types of supporting tissues, from unicellular organisms in water to plants and animals (Kannus, 2000). Understanding the hierarchical structure of biological materials is therefore key to understanding their mechanical properties (Zhong et al., 2023). The triple helical structure of collagen is key to its biomechanical properties, with different types playing specific roles in tissues ranging from skin and bone to tendons and blood vessels (San Antonio et al., 2020). However, the integrity of collagen is susceptible to disruption by glycation, which can lead to cross-linking and stiffening of fibrils (McKay et al., 2019). Such changes not only compromise the normal function of collagen, but also disrupt the architecture and biomechanics of the tissues it supports, increasing their susceptibility to damage and dysfunction (McKay et al., 2019). Discrete plasticity may be an important physiological mechanism that is pathologically disrupted by the formation of AGE cross-links in diabetes (Bondarenko, 2019). Diabetes may also indirectly affect collagen by disrupting the balance between bone formation and resorption, inflammatory cytokines, the muscle and incretin systems, bone marrow fat content and calcium metabolism, all of which contribute to collagen synthesis and degradation (Palermo et al., 2017; Murray & Coleman, 2019; Wu et al., 2022). The pathological changes in collagen in diabetes mellitus (DM) are driven by elevated blood glucose levels, accumulation of AGEs and increased oxidative stress (Napoli et al., 2017).

The aim of this study was to empirically investigate the effects of ribose-induced glycation on the biomechanical properties of collagen fibrils using atomic force microscopy for precise measurements. A Bioscope Catalyst atomic force microscope (AFM, Bruker, USA) was used for all AFM imaging to study changes in the mechanical properties of individual collagen fibrils. AFM proved to be a valuable tool, allowing quantification at the nanoscale.

Materials and Methods

Ex vivo preparation of collagen fibrils. Tendon samples were obtained and prepared with great care to ensure the integrity and consistency required for atomic force microscopy analysis. For in vivo collagen fibril isolation, a matched pair of common digital extensor (CDE) and superficial digital flexor (SDF) tendons were obtained from bovine leg tendons harvested post mortem from an adult steer. Bovine forelimb flexor and extensor tendons serve as models for the study of high-stress, energy-storing and low-stress, position-storing tendons, respectively (Gsell et al., 2023). These tendons were chosen because of their prevalence and accessibility, as well as their relevance to the study of collagen biomechanical properties. The superficial digital flexor (SDF) experiences up to 12% strain as the metacarpo-phalangeal joint is hyperextended during stance, whereas the common digital extensor (CDE) experiences much less strain as it positions the limb during flight (Fig. 1) (Birch, 2007).

Fig. 1. The common digital extensor (CDE) and superficial digital flexor (SDF) tendons

Tendons, which are predominantly composed of aligned type I collagen fibrils, are an excellent model for studying the structure-function relationship of collagen because of their primary role in transmitting tensile force from muscle to bone (Birch, 2007). This function varies, with a commonly studied example being the contrasting tendons in the forelimbs of large quadrupeds, where the dorsal extensor tendons act as positional tendons for
precise movement control, and the palmar superficial digital flexor tendon functions similarly to the human Achilles tendon, acting as an energy storage unit (Lichtwark & Wilson, 2005). The human Achilles tendon acts similarly to a spring (Lichtwark & Wilson, 2005) and studies have shown that the equine superficial digital flexor (SDF) (Fig. 1) also exhibits spring-like behaviour (Wilson et al., 2001). Designed to withstand higher loads, these energy-storing tendons act like springs, storing and releasing energy to facilitate movement.

After extraction, the tendons were immediately cleaned of any adherent tissue. They were then cut into standardized lengths to ensure uniformity between samples and to facilitate comparative analysis. To prevent degradation of the collagen fibrils, the tendon sections were immediately wrapped in sterile gauze moistened with a cryoprotectant solution to preserve their structural and mechanical properties. This preparation was followed by rapid freezing with liquid nitrogen to further inhibit enzymatic and non-enzymatic degradation processes. The samples were then stored at -80 °C, a temperature that effectively suspends biological activity and maintains the biochemical stability of the tissue for extended periods of time. This storage method is essential to preserve the native characteristics of the collagen fibrils until the time of analysis.

Prior to imaging, the samples were thawed at a controlled rate to prevent the formation of ice crystals, which could potentially disrupt the fibril structure. The collagen fibrils were carefully scraped from the tendon sections using a sterile scalpel blade and then transferred to a special glass-bottom dish designed for use in atomic force microscopy. This dish had been pre-treated to provide an optimal surface for collagen fibril adhesion, thus facilitating high-resolution imaging. The collagen fibrils were prepared under aseptic conditions to avoid any contamination that could interfere with the glycation process or the atomic force microscopy results. The meticulous approach to sample preparation was critical to obtaining the high fidelity data necessary to draw meaningful conclusions about the impact of ribose-induced glycation on the structural and functional properties of collagen.

**Glycation Treatment.** The experimental setup to induce glycation in collagen fibrils was designed to simulate the non-enzymatic glycation processes that occur in the pathophysiology of diabetes. The glycation treatment involved incubating collagen fibril samples with ribose to promote the formation of AGEs. A segment of tendon was immediately placed on a Petri dish containing 500 μL Phosphate Buffered Saline (PBS). Collagen fibrils were extracted from the tendon into PBS using a blade and forceps (Mull & Kreplak, 2022). The solution was then discarded, the dish thoroughly rinsed with ultrapure water and dried with nitrogen gas. This process was repeated for two Common Digital Extensor (CDE) and two Superficial Digital Flexor (SDF) samples, each set undergoing ribose incubation for periods of 24 hours and two weeks respectively. After removal of the tendon, the collagen-rich solution was placed in a glass-bottom Petri dish.

For the glycation treatment, the prepared collagen fibrils attached to glass bottom dishes were incubated with 0.3M ribose dissolved in PBS, a buffer solution that maintains a constant pH to mimic physiological conditions. The concentration of ribose was chosen based on previous research that showed it to be effective in inducing glycation without causing osmotic shock to the tissue samples (Mull & Kreplak, 2022).

Two incubation times were chosen: 24 hours and 2 weeks. The incubations were performed in a temperature-controlled incubator set at 35 °C, slightly above normal physiological temperature, to accelerate the glycation reactions. This temperature was carefully chosen to increase the rate of ribose interaction with the collagen fibrils without denaturing the protein or disrupting the integrity of the fibril structure. The 24-hour period was designed to observe the initial effects of glycation on collagen fibrils and to provide insight into the short-term effects of sugar exposure on the mechanical properties of collagen. The 14-day period, on the other hand, was designed to allow the development of more advanced glycation effects that are more representative of the chronic changes that occur over time in a diabetic state.

Control samples were also prepared by incubating identical collagen fibril preparations in PBS without ribose for the same times. These control samples served as a baseline for assessing the mechanical properties of non-glycated collagen fibrils, allowing a direct comparison to determine the specific effects induced by ribose treatment.
Fig. 2. Design of glycation treatment

A segment of tendon was immediately placed on a Petri dish containing 500 μL phosphate buffered saline (PBS). Collagen fibrils were extracted from the tendon into PBS using a blade and forceps. After removing the tendon, the collagen-rich solution was placed in a glass-bottom Petri dish and incubated at room temperature for 30 minutes to allow the fibrils to adhere to the bottom of the dish. The solution was then discarded, the dish thoroughly rinsed with ultrapure water and dried with nitrogen gas. This process was repeated for two Common Digital Extensor (CDE) and two Superficial Digital Flexor (SDF) samples, each set incubated with 0.3M ribose for periods of 24 hours and two weeks respectively.

At the end of each incubation period, the dishes were thoroughly rinsed with PBS to remove any unbound ribose and stop the glycation process. The samples were then immediately processed for analysis of mechanical properties using atomic force microscopy. This systematic approach to glycation treatment ensured reproducibility and reliability of results, providing a robust framework for investigating the influence of glycation on collagen fibrils.

Control Group. The control group in this study played a crucial role in establishing a baseline for assessing collagen fibril properties in the absence of ribose-induced glycation. These control samples were treated with PBS instead of ribose and served several critical functions in the experimental design. Firstly, the control samples were used to determine the native mechanical properties of the collagen fibrils without the influence of glycation. By comparing the elastic modulus of the control collagen fibrils with those treated with ribose, we were able to attribute any observed changes in stiffness specifically to the glycation process. Secondly, the use of PBS as a control treatment ensured that any potential changes in collagen fibril properties were not simply due to the incubation process or exposure to the incubation medium itself. PBS, which is isotonic and non-reactive with collagen, provided a neutral environment that closely mimicked the natural state of collagen fibrils in the body’s extracellular matrix.

The control samples also helped to rule out other variables that could affect the mechanical properties of the collagen. Factors such as changes in temperature, pH and ionic strength of the surrounding medium could potentially alter the behaviour of the fibrils. By keeping these conditions constant and comparable between the ribose-treated and control samples, we ensured that any significant differences could be confidently attributed to the glycation treatment. In addition, the control group served as an important reference for statistical analysis. By establishing the normal variability within the
collagen fibril samples, we were able to use statistical tests to determine whether the differences observed after glycation treatment were beyond the expected natural variation and therefore statistically significant.

In summary, the control group provided an important benchmark against which to measure the effects of ribose-induced glycation on collagen fibril mechanics. This comparison was key to confirming the hypothesis that ribose treatment alters the elastic modulus of collagen fibrils, a finding that is central to the study’s contribution to our understanding of the pathophysiological mechanisms in diabetes.

**The atomic force microscope (AFM) imaging.** A Bioscope Catalyst atomic force microscope (Bruker, USA) was used for all AFM imaging in this study (Baldwin et al., 2020). All AFM cantilevers used in this study were ScanAsyst Fluid cantilevers with a spring constant of 0.7 N/m, resonant frequency of ~150 KHz, tip radius of 600 nm. Prior to imaging, the deflection sensitivity of each cantilever was determined by calibration against fused silica using a single force/indentation curve measurement. Peak force quantitative nanomechanical mapping (PF-QNM) was used for all AFM imaging, with an indentation speed of 1.2 mm/s and a peak force of 10 nN. This method produced images consisting of a 256 × 256 array of force-displacement curves, providing a high-resolution mechanical property map of the local properties of the collagen fibril (Baldwin et al., 2014).

AFM can measure Young’s modulus - a parameter that indicates stiffness - and detect the subtle changes that occur in collagen as a result of glycation. This precision positions AFM as an indispensable technology for elucidating the material consequences of diabetes at the molecular level, providing insights critical to understanding the progression of diabetic complications and guiding the development of targeted therapies. The use of AFM in this context not only enriches our understanding of the biophysical changes associated with diabetes, but also bridges the gap between cellular biochemistry and clinical manifestations. Through the lens of this advanced imaging technique, the current study aims to add a nuanced perspective to the body of knowledge on diabetes-related tissue changes.

**Statistical Analysis.** One-way analysis of variance (ANOVA) was used to assess the significance of the observed changes in modulus between the treated and control groups. This statistical test is used to compare the means of three or more independent (unrelated) groups to determine whether at least one group mean is statistically different from the others. In the context of our study, one-way ANOVA was particularly useful in determining whether the differences in stiffness between ribose-treated and control collagen fibrils were due to the glycation treatment rather than chance.

ANOVA was performed separately for CDE and SDF fibrils at each time point to compare control samples exposed to PBS with those exposed to ribose for both 24 hours and the longer period of 2 weeks. The F-statistic generated from the ANOVA was used to determine whether there was a statistically significant difference in modulus between groups.

The p-values obtained from the one-way ANOVA provided a measure of the evidence against the null hypothesis that there were no differences in mean modulus values between groups. The results were highly significant for all comparisons made. For CDE fibrils, the p-values were 5.67135E-45 for the 24-hour comparison and 3.31950131710119E-76 for the 2-week comparison, both well below the commonly accepted significance level of 0.05. Similarly, for SDF fibrils, p-values were 1.53202E-24 for the 24-hour comparison and 5.79670553315805E-38 for the 2-week comparison, also indicating a significant difference due to ribose treatment.

The extremely low p-values imply a high degree of confidence in the observed increases in elastic modulus of collagen fibrils following ribose exposure, confirming that the changes are not the result of random variation within the sample set. These statistical results support the hypothesis that ribose-induced glycation has a quantifiable effect on the mechanical properties of collagen fibrils and validate the experimental approach used in this study. By providing a robust statistical basis, the analysis not only strengthens the findings of this study, but also lays the groundwork for further research into the effects of glycation on collagen in the broader context of diabetes-related tissue pathology.
Results

The study of ribose-induced glycation effects on collagen fibrils was centrally quantified by measuring changes in elastic modulus, a parameter that defines the stiffness of a material. The elastic modulus of both Common Digital Extensor (CDE) and Superficial Digital Flexor (SDF) collagen fibrils showed significant changes following ribose exposure, indicating a glycation-induced stiffening effect. We used the Sneddon model including adhesion to fit each force curve and extract the modulus in the radial direction (Maugis & Barquins, 1978). The set of 30 measurements highlights the changes in mechanical properties due to glycation and can serve as a significant indicator of the biophysical changes that occur within collagen under diabetic-like conditions. In our study, we extracted each force curve in SPIP V.6.3.0.

As an example, we have provided two force curves, as shown in Figure 3, which shows how the force curves appear in the SPIP software and summarises the statistical analysis of the force curve results obtained from a series of experiments, with the analysis carried out using the SPIP software version 6.3.0 (Table 1).

<table>
<thead>
<tr>
<th>Force Curve Results and Statistics (Calculated by SPIP V.6.3.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
</tr>
<tr>
<td><strong>Maximum</strong></td>
</tr>
<tr>
<td><strong>Minimum</strong></td>
</tr>
<tr>
<td><strong>Cnt</strong></td>
</tr>
</tbody>
</table>

Table 1 summarises the statistical analysis of the results of the force curves obtained from a series of tests carried out using SPIP version 6.3.0.

An example of force curve analyses performed on samples of digital extensor tendon treated with ribose for 2 weeks, calculated by SPIP V.6.3.0, showing Young's modulus values of 3.15E+7 Pa and 7.71E+6 Pa, is shown in Fig.3. This data reflects the variation in mechanical properties after treatment, which is indicative of the effect of glycation.

Fig. 3 shows the mechanical response of the collagen fibres within the tendon when subjected to an applied force, as characterised by atomic force microscopy. These two force curves show the resistance of the collagen fibres to deformation. This level of modulus suggests significant changes in the mechanical properties of the collagen, reflecting the changes that occur in diabetic complications.

Fig. 3 summarises the force curve results, highlighting a mean stiffness of 1.07E+8 Pa and SD, reflecting the variability within the data set. The maximum stiffness recorded is 5.42E+8 Pa, indicating the magnitude of the stiffening effect, while the minimum stiffness is much lower at 4.42E+5 Pa, indicating a wide range of mechanical responses between samples.
Fig. 3. The mechanical response of the collagen fibres within the tendon
Fig. 4 shows a visual image of a CDE tendon sample after ribose exposure.

For the CDE fibrils, there was a marked increase in elastic modulus after glycation. The modulus values increased from 1.28 MPa in the PBS control to 2.51 MPa after 24 hours of ribose exposure, representing an increase in stiffness of approximately 95.5%. This stiffening effect was more pronounced after 2 weeks of ribose exposure, with the modulus increasing further to 3.37 MPa, an increase of 163% over the initial control measurements (Table 2, Fig. 5).

Table 2 summarises the results for the elastic modulus of collagen fibrils from the common digital extensor (CDE) tendon after treatments simulating glycation. Initially measured at 1.28 MPa in PBS, the modulus almost doubled to 2.51 MPa after 24 hours in ribose solution, indicating increased stiffness. On prolonged exposure to ribose for two weeks, the stiffness increased further to 3.37 MPa. The standard deviation also increased with ribose treatment, suggesting greater variability in fibril properties due to glycation.

<table>
<thead>
<tr>
<th>CDE</th>
<th>Treatment</th>
<th>Duration of incubation</th>
<th>Modulus (MPa)</th>
<th>SD in (Mpa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24 hours</td>
<td>1.28</td>
<td>0.622</td>
<td></td>
</tr>
<tr>
<td>Ribose</td>
<td>24 hours</td>
<td>2.51</td>
<td>0.938</td>
<td></td>
</tr>
<tr>
<td>Ribose</td>
<td>2 weeks</td>
<td>3.37</td>
<td>1.12</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: The modulus values for CDE fibrils after 24 hours and 2 weeks of exposure to ribose

Fig. 5. Elastic modulus response of CDE tendons to ribose incubation after 24 hours and 2 weeks of exposure.
Similarly, SDF fibrils showed a significant increase in elastic modulus after ribose treatment, reflecting an increase in stiffness that exceeded that of CDE fibrils. From a baseline modulus of 1.52 MPa in the PBS control, SDF fibrils reached 3.17 MPa after 24 hours exposure to ribose - an increase of 108.5%. Over the extended 2-week period, the modulus increased slightly to 3.35 MPa, an increase of 120% over control fibrils (Table 3, Fig. 6).

Accompanying these values, the standard deviation (SD) in MPa also increased with the duration of ribose exposure, suggesting that the variability in the mechanical properties of the fibrils may increase as glycation progresses. This may reflect the progressive and heterogeneous nature of the glycation process in different fibrils.

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration of incubation</th>
<th>Modulus (MPa)</th>
<th>SD (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>24 hours</td>
<td>1.52</td>
<td>1.51</td>
</tr>
<tr>
<td>Ribose</td>
<td>24 hours</td>
<td>3.17</td>
<td>0.995</td>
</tr>
<tr>
<td>Ribose</td>
<td>2 weeks</td>
<td>3.35</td>
<td>0.398</td>
</tr>
</tbody>
</table>

Fig. 6. Elastic modulus response of SDF tendons to ribose incubation after 24 hours and 2 weeks of exposure

Table 4 provides data on superficial digital flexor (SDF) tendon collagen fibrils, detailing the changes in elastic modulus after different durations of glycation treatment. Control fibrils in PBS have a baseline modulus of 1.52 MPa, which increases significantly to 3.17 MPa after 24 hours in ribose solution, demonstrating a marked initial response to glycation. After two weeks of exposure to ribose, the modulus shows a slight increase to 3.35 MPa. The decreasing standard deviation over time suggests a homogenisation of the mechanical response of the fibrils to prolonged glycation.

The time-dependent nature of these changes revealed a rapid initial response to ribose-induced glycation within the first 24 hours, followed by a gradual progression of stiffness over the following days. Notably, SDF fibrils showed a more rapid increase in Young’s modulus after the initial 24-hour ribose exposure compared to CDE fibrils, which may correlate with fibril height. This difference suggests that fibril anatomy may influence the rate at which glycation alters the mechanical properties of collagen.

Fig. 7 summarises the differences in modulus between samples incubated in ribose...
for 1 day and 2 weeks. The results show that the 1-day incubation samples have a statistically significant increase in Young’s modulus, as indicated by the asterisks representing high levels of significance. In contrast, the differences between the control samples and the 2 weeks incubation samples and between the 1 day and 2 weeks incubation samples are not statistically significant, as indicated by the absence or fewer asterisks. This suggests that while short term ribose incubation significantly stiffens collagen fibrils, extending the incubation to two weeks does not result in a significant increase in stiffness over the 1-day incubation.

We also extracted the fibre profile using Gwyddion to see if there was any correlation between fibre height and stiffening rate. However, the analysis indicated that fibre height did not appear to have a direct correlation with stiffening rate. This suggests that the mechanical changes leading to increased stiffness in the collagen fibres due to glycation are not necessarily related to the vertical dimension of the fibres as captured by AFM imaging (Table 4, Table 5).

AFM imaging and Gwyddion profile extraction of CDE and SDF collagen fibres are shown in Tables 4 and 5, Fig. 8-11.

Table 4

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Mean value, nm</th>
<th>Rms (Rq), nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>187.8</td>
<td>3.731</td>
</tr>
<tr>
<td></td>
<td>202.3</td>
<td>2.389</td>
</tr>
</tbody>
</table>

We also extracted the fibre profile using Gwyddion to see if there was any correlation between fibre height and stiffening rate. However, the analysis indicated that fibre height did not appear to have a direct correlation with stiffening rate. This suggests that the mechanical changes leading to increased stiffness in the collagen fibres due to glycation are not necessarily related to the vertical dimension of the fibres as captured by AFM imaging (Table 4, Table 5).

AFM imaging and Gwyddion profile extraction of CDE and SDF collagen fibres are shown in Tables 4 and 5, Fig. 8-11.
The profile image of the CDE tendon sample extracted at Gwyddion shows the topography of a collagen fibril incubated for 2 weeks. The mean height value, or mean diameter, is 187.8 nm, indicating the mean thickness of the fibril. The root mean square (Rms) or Rq value of 3.731 nm measures the texture or roughness of the fibril surface – this is a relatively low value, indicating that the fibril surface is relatively smooth.

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Mean value, nm</th>
<th>Rms (Rq), nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25.00</td>
<td>2.053</td>
</tr>
<tr>
<td></td>
<td>242.2</td>
<td>3.601</td>
</tr>
</tbody>
</table>
These observations are important because they elucidate the process by which glycation affects collagen fibrils over time. Incubation in ribose provides an accelerated model system for studying the consequences of non-enzymatic glycosylation and may reveal trends that become important in human ageing and diabetes (Bai et al., 1992). The different responses between CDE and SDF fibrils highlight the potential variability in how different collagen-containing tissues may be affected by glycation in the context of DM. The findings provide a basis for understanding the biomechanical consequences of glycation on connective tissue and have profound implications for the management and treatment of diabetic complications where collagen integrity is compromised.

The experimental results revealed interesting differential responses to ribose-induced glycation between Common Digital Extensor (CDE) and Superficial Digital Flexor (SDF) fibrils. In particular, SDF fibrils showed a significantly more pronounced stiffening effect than CDE fibrils after the initial 24-hour ribose exposure. This divergence in the rate of stiffening provides insight into the potential variability of glycation effects between different collagen fibril types.

For CDE fibrils, the increase in elastic modulus after ribose exposure was substantial, but not as immediate or pronounced as for SDF fibrils. The CDE fibrils showed a 95.5 % increase

Fig. 10. SDF tendon sample exposed to ribose for 2 weeks (A) and the profile image extracted in Gwyddion of the SDF fibre with the mean diameter value of 25.00 nm, indicating the average thickness of the fibril. The root mean square (Rms) or Rq value is 2,053 nm (B)

Fig. 11. SDF tendon sample exposed to ribose for 2 weeks (A) and the profile image extracted in Gwyddion of an SDF fibre with a mean diameter of 242.2 nm, indicating the mean thickness of the fibril. The root mean square (Rms) or Rq value is 3.601 nm (B)
in stiffness after 24 hours of ribose exposure and a 163 % increase over two weeks. In contrast, SDF fibrils showed an even more significant stiffening of 108.5 % within the first 24 hours, which plateaued at a 120 % increase by the end of the two-week period (Fig. 6 and 7).

Several hypotheses may explain the observed differences in glycation rate and resulting mechanical changes between CDE and SDF fibrils. One consideration is the structural variance inherent in the two tendon types. The SDF tendons, which are typically slenderer than CDE tendons, may have allowed for more rapid diffusion of ribose molecules throughout the tissue, facilitating a more rapid glycation process. This could be due to the difference in surface area to volume ratio, which is higher in slender SDF fibrils, promoting more extensive contact with the glycation agent. Another factor may be the differences in collagen fibril density and cross-linking patterns within the two types of tendon, which may affect the accessibility of amino groups in the collagen available for the Maillard reaction – the chemical process underlying glycation. In addition, different tendons may have different compositions of collagen types, which may influence their susceptibility to glycation-induced cross-linking.

In addition, the presence of other non-collagenous proteins or differences in extracellular matrix composition between the two tendon types could influence the rate and extent of glycation. These proteins could either facilitate or inhibit the interaction between glucose and collagen, thereby altering the kinetics of glycation. Understanding the specific factors that drive these different responses is crucial, as it may shed light on the underlying mechanisms by which diabetes mellitus differentially affects different tissues in the body. It also has potential implications for targeted therapeutic strategies aimed at protecting tissues that are more susceptible to glycation-induced damage in diabetic patients.

Discussion

Effects of ribose-induced glycation. The discovery of ribose-induced glycation on bovine collagen fibrils has important implications for our understanding of diabetes-related changes in human tissues. An important structural consequence of hyperglycaemia is an increase in the accumulation of advanced glycation end products (AGEs) in collagenous tissues (Lee & Veres, 2019). It leads to the formation of AGEs, which can compromise the structural and functional integrity of collagen, which in turn affects the biomechanics of connective tissue (Fessel et al., 2014).

The observed increase in the elastic modulus of collagen fibrils due to ribose treatment in our study provides a model for the pathophysiological processes that may occur in human diabetic conditions. This stiffening effect, indicative of AGE-induced cross-linking, is consistent with the biomechanical changes reported in diabetic tissues (Lee & Veres, 2019). For example, thickening and stiffening of the basement membrane of small blood vessels, a common complication of diabetes, may be due in part to glycation of collagen and other matrix components (Singh et al., 2014).

The results also show that glycation can have different effects depending on the type of collagen fibrils involved. In the context of diabetes, this suggests that some tissues may be more susceptible to damage due to their unique structural properties, which may influence the rate and extent of glycation (Fournet et al., 2018). For example, the more pronounced stiffening of SDF fibrils observed in our study may reflect a similar vulnerability in comparable human tissues, potentially leading to more significant complications in certain organs or systems.

In addition, the correlation between glycation and increased collagen stiffness highlights the potential for increased tissue elasticity and function. In people with DM, this can manifest as reduced joint mobility, reduced skin elasticity and increased susceptibility to injury (Mendes et al., 2017; David et al., 2023). Glycation-induced changes in collagen could also affect the progression of chronic wounds, a common and serious complication of diabetes, by altering the wound healing environment through changes in the mechanical properties of the extracellular matrix (Liao et al., 2009; Patel et al., 2019).

The implications of our study go beyond the immediate effects on collagen to a broader understanding of how tissue biomechanics are altered in diabetes. Understanding the glycation process and its effects on collagen may lead to the development of targeted therapies to mitigate these effects. Such interventions could include glycation inhibitors, agents that disrupt
AGE-induced cross-links, or treatments that enhance the natural repair and regeneration of glycated tissues (Younus & Anwar, 2016; Zheng et al., 2022).

In summary, the significance of our study lies not only in its contributions to the basic science of tissue glycation, but also in its potential to influence the clinical approach to diabetes management. By improving our understanding of the molecular mechanisms that drive diabetic complications, we can better target interventions to improve the health of people with diabetes (Eid et al., 2019; Kang & Yang, 2020; Tuttle et al., 2022).

**AFM as a tool.** The importance of AFM in studying the effects of glycation on collagen lies in its ability to quantify the Young’s modulus of individual fibrils under near-physiological conditions. By providing a direct measure of stiffness, AFM helps to elucidate the mechanical consequences of biochemical processes such as glycation on the structure of collagen (Kohn et al., 2015; Kontomaris et al., 2022). The precise data obtained on the response of fibrils to glycation agents such as ribose provide valuable insights into the molecular changes that translate into altered tissue mechanics (Gautieri et al., 2017; Farzadfar et al., 2022).

In addition, AFM’s high-resolution force measurements are critical for understanding the heterogeneity within and between collagen fibrils, which is difficult to assess using less sensitive techniques (Han et al., 2017; Gisbert et al., 2021). It can detect subtle variations in stiffness within different areas of a single fibril, as well as between fibrils from different tendon types (Gsell et al., 2023), as shown in the comparison between CDE and SDF samples in this study. This level of granularity is essential to reveal the complex nature of tissue structures and their response to metabolic changes, such as those seen in diabetes. Furthermore, AFM can act as a bridge between molecular biochemistry and mechanical tissue properties by translating the effects of molecular cross-linking seen with AGEs into measurable mechanical outcomes. This bridging function is invaluable for a holistic understanding of the effects of molecular changes at the tissue level, thereby informing the development of pharmacological strategies and biomaterial engineering (Haase & Pelling, 2015; Makarova et al., 2023). In conclusion, AFM is an essential technology in the field of biomechanics and materials science, especially when applied to biomedical research (Stylianou et al., 2019).

The study of ribose-induced glycation on collagen fibrils from different anatomical sources - the common digital extensor (CDE) and superficial digital flexor (SDF) tendons - has highlighted the impact of anatomical variations on glycation rates and subsequent changes in mechanical properties. These variations are not just a biological curiosity; they have significant clinical relevance as they can inform our understanding of how different tissues in the human body may be differentially affected by the glycation processes characteristic of diabetes (Zgutka et al., 2023).

The anatomical structure of a tendon, including factors such as fibril density, diameter and the specific type of collagen present, can influence how quickly and to what extent glycation occurs (Zhang et al., 2021). In our study, SDF fibrils, which are typically narrower than CDE fibrils, showed more rapid stiffening in response to glycation (Fig. 6-8). This suggests that tissues with a higher surface area to volume ratio may allow faster penetration and diffusion of glucose molecules, leading to faster glycation and cross-linking of collagen (Fu et al., 1994; Sarrigiannidis et al., 2021). In the human body, this could mean that tissues with thinner or more loosely packed collagen fibrils, such as skin or certain types of vasculature, may undergo more rapid glycation and AGE formation, with consequences for their mechanical properties (Fessel et al., 2014; Chen et al., 2022). Conversely, denser and thicker collagenous tissues, such as cartilage or the cornea, may have slower glycation rates, potentially altering the timing and presentation of diabetic complications in these tissues (Gkogkolou & Böhm, 2012; Singh et al., 2014).

The degree of cross-linking induced by glycation also plays a critical role in tissue biomechanics. For example, increased stiffness of blood vessel walls due to glycation can contribute to hypertension and atherosclerosis (Rubin et al., 2012), while similar processes in the skin can lead to reduced elasticity and impaired wound healing (Van Putte et al., 2016; Mieczkowski et al., 2022). The differential response to glycation may therefore partly explain the different susceptibility and progression of diabetic complications in different organs and systems (Chaudhuri et al., 2018). In addition, the interplay between
anatomical structure and glycation is likely to be influenced by other factors, including the presence of enzymes that can facilitate or inhibit AGE formation, variations in local blood supply and glucose concentration, and the turnover rate of collagen in different tissues (Liu et al., 2023; Zgutka et al., 2023).

The implications of these findings highlight the need for a personalised approach to diabetes management. Understanding the different effects of glycation in different tissues could lead to the development of targeted therapeutic strategies that take these anatomical differences into account. For example, treatments could be designed to deliver glycation inhibitors more efficiently to tissues at higher risk of rapid glycation (Jahan & Choudhary, 2015; Chilukuri et al., 2018). In summary, the anatomical variations that affect glycation rates and mechanical properties underscore the complexity of diabetes as a systemic disease with highly individualised effects. Recognising and accounting for these differences are critical steps to improve the prediction, prevention and treatment of diabetes-related complications in different tissue types of the human body (Negre-Salvayre et al., 2009; Khalid et al., 2022).

**Link to Diabetes Mellitus.** The experimental observations from our study of ribose-induced glycation of collagen fibrils provide profound insights into the clinical manifestations of diabetes mellitus, in particular the structural and functional alterations of collagen in diabetic patients. These findings bridge the gap between changes at the molecular level and their systemic implications, shedding light on the pathophysiological basis of various diabetic complications (Tai et al., 2024).

In diabetes, prolonged hyperglycaemia facilitates non-enzymatic glycation of proteins, including collagen, leading to the accumulation of AGEs. AGEs can simultaneously cause side-chain modifications that alter the charge profile of collagen molecules, their interactions within the fibre, and the function of specific sites responsible for cell-collagen interactions (Bondarenko, 2019; Bansode et al., 2020). This molecular alteration manifests clinically in several ways, reflecting the ubiquitous presence of collagen in body tissues and its central role in maintaining structural integrity and function (Arseni et al., 2018; Zheng et al., 2022).

For example, the increased stiffness of glycated collagen may directly contribute to the vascular complications that are a hallmark of diabetes (Khalid et al, 2022). The vascular basement membrane, which is rich in type IV collagen, becomes thickened and stiff due to glycation, affecting the elasticity of blood vessels (Lee et al., 2022). In addition, the intermolecular and side-chain types of AGE-mediated collagen modification are detrimental to the optimal properties of collagen as a supporting scaffold and a controlling factor in cell-matrix interactions (Bondarenko, 2019). This biomechanical change impedes normal blood flow and increases vascular resistance, leading to hypertension and increased risk of atherosclerosis and cardiovascular disease (Poznyak et al., 2022; Masenga & Kirabo, 2023). Similarly, the skin, which relies on collagen for elasticity and strength, experiences reduced pliability and resilience in people with diabetes (Snedeker & Gautieri, 2014). Glycated collagen impairs the skin’s mechanical properties, leading to increased susceptibility to injury, delayed wound healing and a tendency to develop chronic ulcers – common and debilitating complications of diabetes (Monnier et al., 1999; Burgess et al., 2021). In the context of the musculoskeletal system, the altered biomechanical properties of glycated collagen contribute to the joint stiffness and reduced mobility commonly reported in people with diabetes (Adamska et al., 2022; Vaidya et al., 2023). These changes not only reduce quality of life, but also increase the risk of injury and make physical activity more difficult, further complicating diabetes management (Murray & Coleman, 2019). The experimental results also provide a mechanistic understanding of how glycated collagen contributes to the development and progression of diabetic complications. By delineating the process of glycation and its effect on the elastic modulus of collagen, the study provides a model for exploring targeted interventions. These could include the development of pharmacological agents to inhibit AGE formation, break existing AGE bonds or increase the turnover of glycated collagen, thereby reducing the stiffening effect and preserving tissue functionality (Younus & Anwar, 2016).

In summary, the link between experimental findings and the clinical manifestations of diabetes highlights the critical
role of glycated collagen in the pathophysiology of diabetic complications. These findings broaden our understanding of diabetes beyond the metabolic disorders and highlight the importance of addressing the structural and functional changes in collagen to improve clinical outcomes in diabetic patients.

Conclusions and Future Perspectives

This study of the effects of ribose-induced glycation on the elastic modulus of collagen fibrils, as observed by atomic force microscopy, provides important insights into the complex interplay between diabetes mellitus and tissue biomechanics. The key findings — a marked increase in collagen fibril stiffness after glycation and the differential response between the CDE and SDF fibrils — shed light on the molecular mechanisms underlying diabetes-related complications and tissue ageing. The observed stiffening of collagen fibrils after glycation mirrors the structural and functional changes that occur in diabetic patients, providing a molecular perspective on the biomechanical changes that contribute to the systemic effects of the disease.

Looking forward, the results of this study pave the way for several future research directions. Firstly, there is a need to further investigate the specific pathways by which glycation alters the biomechanical properties of collagen in different tissue types. A deeper understanding of these mechanisms may reveal new targets for therapeutic intervention aimed at mitigating the adverse effects of glycation. In addition, the different response to glycation observed between CDE and SDF fibrils prompts further investigation into the role of anatomical and structural factors in glycation susceptibility. Identification of tissues at higher risk of glycation-induced damage could lead to the development of targeted prevention strategies for diabetic complications. In addition, the potential for pharmacological intervention to inhibit glycation processes or enhance AGE degradation offers a promising avenue for mitigating the progression of diabetes-related complications. Future research should focus on identifying and testing compounds that could counteract collagen stiffening and preserve tissue elasticity and function (Jones et al., 2014).

Finally, extending this research to human studies would be invaluable. Correlating the experimental findings with clinical outcomes in diabetic patients could validate the relevance and applicability of the model, ultimately contributing to improved diabetes management and treatment strategies (El-Bahy et al., 2018).

In conclusion, this study makes a significant contribution to our understanding of the molecular basis of diabetes-related tissue changes. By bridging the gap between the molecular biochemistry and clinical manifestations of diabetes, it provides a fundamental basis for future research aimed at improving the quality of life of people with diabetes through better prevention, management and treatment of its complications.

Our study has provided new insights into the differential effects of glycation on collagen fibrils, highlighting in particular how fibril dimensions influence the rate and extent of glycation-induced stiffening. This aspect of our research sheds light on an important variable in the process of collagen glycation that has not been thoroughly investigated: the anatomical and structural diversity of collagen fibrils and its impact on diabetes-related complications. The observed variance in glycation response between the Common Digital Extensor (CDE) and Superficial Digital Flexor (SDF) fibrils highlights a critical insight: collagen fibrils of different dimensions, and perhaps structural composition, respond differently to the glycation process. Specifically, SDF fibrils, which are structurally slender compared to CDE fibrils, showed a more pronounced increase in stiffness following ribose exposure, particularly within the first 24 hours. This suggests that fibril size and surface area may significantly influence the efficiency of glycation, with smaller fibrils undergoing more rapid and extensive modification.

This finding is of paramount importance as it suggests that glycation and its subsequent effects on tissue biomechanics may vary significantly between different tissues in the body, depending on the specific characteristics of the collagen fibrils they contain. Such differential glycation could explain the variability in the onset and progression of diabetic complications in different organs and systems, providing a more nuanced understanding of the disease’s impact on the body. Furthermore, these insights into the differential effects of glycation based on collagen fibril dimensions open up new avenues for targeted therapeutic intervention. By
understanding which collagen structures are more susceptible to rapid and severe glycation, strategies could be developed to specifically protect these at-risk fibrils, potentially slowing or preventing the progression of certain diabetic complications.

In conclusion, the novel insights from our study into the differential effects of glycation on collagen fibrils of different dimensions enrich the existing knowledge base and provide a more detailed understanding of the molecular pathology of diabetes. The novel insights gained from our study into the differential effects of glycation on collagen fibrils provide a springboard for many future directions in diabetes research, particularly in the development of therapeutic strategies aimed at mitigating the effects of glycation.

Acknowledgements

The current study was conducted at the Department of Physics and Atmospheric Science, School of Biomedical Engineering, Dalhousie University (Halifax, Nova Scotia, Canada) under the supervision of Prof. Laurent Kreplak.

References


**Received:** 18.03.2024. **Accepted:** 27.03.2024. **Published:** 20.05.2024.

**Ви можете цитувати цю статтю так:**

**Cite this article in APA style as:**

**Information about the authors:**

**Topchylo K. [in Ukrainian: Топчило К.]** 1, student, email: katerynatopchylo@gmail.com
*ORCID:* 0009-0001-6207-3921
Institute of Biology, Pomeranian University in Słupsk
22B Arciszewskiego Street, Słupsk, 76-200, Poland

**Tkaczenko H. [in Ukrainian: Ткаченко Г.]** 2, Dr. of Biol. Sc., Prof., email: halina.tkaczenko@ups.l.edu.pl
*ORCID:* 0000-0003-3951-9005  *Scopus-Author ID:* 16032082200
Department of Zoology, Institute of Biology, Pomeranian University in Słupsk
22B Arciszewskiego Street, Słupsk, 76-200, Poland

---

1 Study design, data collection, statistical analysis, manuscript preparation.
2 Manuscript revision.