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Thermal stability of collagen in naturally ageing and in vitro glycated rabbit tissues

Hanna Trębacz[1] · Agnieszka Szczęsna[1] · Marta Arczewska[2]

Abstract

The aim of the work was to compare glycation-related changes in thermal denaturation of collagen in naturally ageing and in vitro ribosylated tissues. Samples of cornea, meniscus and Achilles tendon from young adult and from ageing rabbits were compared. Moreover, in vitro glycated samples (ribose, 100 mg mL^{-1}, 14 days) were prepared for both age groups. Collagen in tissues was characterized in terms of thermal denaturation parameters obtained from differential scanning calorimetry. The level of pentosidine and other fluorescent advanced glycation end products (AGEs) in the papain-digested tissue samples was evaluated using spectrofluorimetry ($\lambda_{ex/em}$: 335/385 and 370/440 nm). In naturally ageing tissues, changes in properties of extracellular matrix collagen expressed in terms of parameters of thermal denaturation and fluorescent AGEs levels were tissue dependent and were related to differences in organization of matrix components. No signs of increased level of AGEs were found in the naturally ageing cornea, unlike in the tendon and meniscus. In vitro ribation proved by gains of fluorescent AGEs affected thermal stability of collagen in all tissues, both in young adult and in the ageing ones. The effects of ribation were considerably greater than the effects of natural ageing. The increase in denaturation temperature was similarly strong in all tissues and did not correlate with the increase in fluorescent AGEs. As a conclusion, parameters of collagen thermal denaturation are tissue and age dependent and an amount of fluorescent AGEs is not the only determinant of increasing thermal stability of glycated collagen.

Keywords Achilles tendon · Cornea · Differential scanning calorimetry · Fluorescent AGEs · Meniscus

Introduction

The main structural protein in mammalian tissues is the fibril forming collagen type I which accounts for around 90% of all body collagens [1]. Formation of all collagen fibres is based on similar biochemical pathways—post-translational modifications of lysine residues [2, 3]. However, both the extent of hydroxylation of lysine in the molecules and the presence of other matrix constituents result in the diversity of structures among collagen based tissues. Even though the primary sequence of a type I collagen molecule is identical in different tissues, a broad variety of possible dimensions and arrangements of fibres results in very different structures, such as tendon, skin, bones, cornea or fibrocartilage [1, 2]. Moreover, the structure of collagenous matrix in the same tissue changes during maturation and ageing as a result of enzymatic and non-enzymatic crosslinking residues. Lysyl oxidase-mediated reactions involving lysine and hydroxylysine residues result in crosslinks which spontaneously stabilize forming a mature collagen network responsible for the functional integrity of the tissue [3, 4]. Non-enzymatic reactions between collagen and sugars result in various advanced glycation end products (AGEs) that form additional intermolecular crosslinks or protein adducts [5]. AGEs accumulate with age and impair both mechanical and biological functions of tissues [4, 6, 7].

Glycation is a natural result of reactions of long-lived proteins with sugars present in tissues, but the process can be markedly enhanced due to diabetes. AGEs were found in all types of connective tissue, both in healthy ageing and

✉ Hanna Trębacz
  hanna.trebacz@umlub.pl

1 Department of Biophysics, Medical University of Lublin, Al. Racławickie 1, 20-059 Lublin, Poland

2 Department of Biophysics, University of Life Sciences in Lublin, Lublin, Poland

in diabetic ones, and it is believed that glycation plays a main role in their ageing and disease-associated dysfunctions [8–12].

Since stability of the triple helical collagen molecule results from heat-labile hydrogen bonds, the overall condition of protein can be studied in terms of thermally induced transformations. Upon heating, these bonds are broken and the molecules unwind resulting in transformation of highly ordered collagen structure into an unstructured coil state [13–15]. Collagen denaturation is greatly influenced by the composition of the molecule, its post-translational modifications, molecular organization and the environment [15, 16]. Conditions in which collagen molecules denature were frequently examined by using differential scanning calorimetry (DSC). Changes in the temperature and enthalpy of denaturation, as well as shape of the curve, were shown to be associated with differences in the post-translational modifications of collagen, and especially, the temperature was proved to be sensitive to the presence of crosslinks [17–21]. Parameters of thermal denaturation are also very sensitive to hydration of collagen fibres [22–24]. The thermal studies of collagens were conducted using model collagen-like peptides [14], collagens extracted from tissues [17, 18, 22, 23, 25] and on tissue collagens in situ [24–32]. Tissues from musculoskeletal system were the most frequently examined [24–26, 28, 29, 32], and thermal parameters of collagen denaturation were shown to be related to different types of the tissues disorders.

Effects of glycation on collagen were studied by means of various in vitro models which try to mimic structural changes of fibrils and to evaluate their effects on tissue properties [20, 21, 25, 33]. An issue still discussed is whether stabilization of collagen fibres by AGEs formation resulting from an excess of sugars follows a similar pattern as that seen in normal ageing [10–12, 21].

The main rationale for our study was questions on relations between a complex structure and composition of tissue extracellular matrix and changes of collagen properties in tissues during ageing. A specific goal of the present work was to compare thermal denaturation of collagen in naturally ageing cornea, meniscus and Achilles tendon of rabbits and the thermal denaturation of these tissues glycated in vitro to study glycation-related changes of collagen properties.

Materials and methods

Samples

Achilles tendons, corneas and menisci of six young adult (8–9 months old) and six ageing (28–34 months old)

Californian rabbits were used in the study. Hindlimbs and heads of the animals were obtained from a local breeder. Three sets of samples were prepared from the tissues of both young adult (Y) and ageing (A) animals: one freshly isolated control for analysis of natural ageing effects (C), one incubated in ribose (R) and one buffer incubated (B). R samples were subjected to in vitro glycation by incubation for 14 days in 100 mg mL^{-1} of ribose in Hanks buffer/ 1.3 mM CaCl$_2$ at 37 °C supplemented with 5 mg of gentamicin for 10 mL as a prevention against bacterial growth. B samples were incubated for the same time period in the same buffer without ribose. The samples were stirred every day, and the solution was exchanged after 5 and after 10 days of incubation. After the incubation period, the samples were washed carefully with distilled water and placed into 0.9% saline.

One half of each sample from the freshly isolated and incubated groups was used for calorimetric measurements and the other halve for spectrofluorimetry. Calorimetric measurements were taken within 1–2 days after harvesting the tissues (Y–C, A–C) or after incubation period (Y–R, A–R, Y–B, A–B). The samples were kept in saline at 4 °C prior to measurements.

Calorimetry

Thermal stability of collagen in samples was evaluated by means of differential scanning calorimetry (DSC). Samples of tissues were drained from excess of water on a filter paper, weighed (5–7 mg) and sealed in hermetic aluminium pans with 20–25 μL of saline. After 1 day of conditioning at room temperature, samples were placed into a calorimeter (Q200, TA Instruments, USA) and scanned from 25 to 90 °C in nitrogen atmosphere at scanning rate of 5 °C min^{-1}. As a reference, a hermetic pan with saline was used. The peak temperature of denaturation at maximum heat absorption (T_m), the change of enthalpy (ΔH) corresponding to the energy absorbed by the tissue during helix-coil transformation of collagen (in joules per gram of the sample) and the width at half-peak height ($\Delta T_{1/2}$) were determined from the curve using a software integrated with the calorimeter (Fig. 1). After the measurement, the pan was opened, the sample drained from excess of saline and dried 48 h in a desiccator at room temperature in order to find a dry mass. Enthalpy of denaturation was recalculated in respect of the dry mass of the sample (ΔH_d).

Spectrofluorimetry

The level of glycation in the tissues was evaluated using spectrofluorimetric method. Because of small amount of tissue material from individual animals, all six samples in

Fig. 1 DSC curves of rabbit tissue samples from the ageing control group
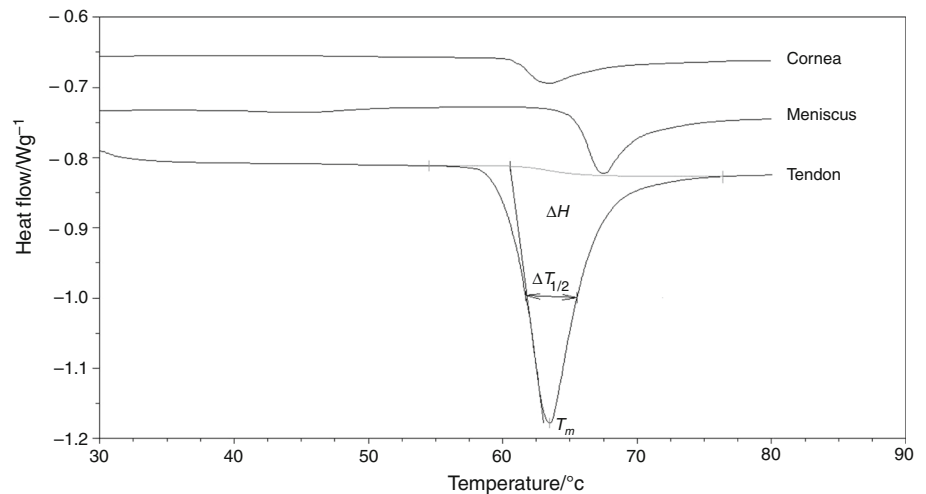
each group were collected together for the analysis. Dried samples were digested in papain (0.4 mg mL^{-1}, from *Papaya latex* from Sigma-Aldrich 10 U mg^{-1}) in 0.1 M sodium acetate buffer (pH 6.5) in 60–66 °C for 2 h. Next, the samples were centrifuged at 25 °C (10 min, 178,600×g). Then, 200 µl of papain digest was taken and added to 1800 µl of the same buffer.

Fluorescence emission spectra of digested samples solutions were measured at $\lambda_{ex/em}$ 335/385 and 370/440 nm with a Cary Eclipse spectrofluorometer from Varian. The excitation and emission slits were set to 5 nm. The results were expressed as fluorescence intensity spectra (in arbitrary units) accounting for fluorescence background. It was widely proved that fluorescence intensity of papain-digested samples at 390–400 nm, after excitation with 335 nm, is related to pentosidine content and the fluorescence at 420–440 nm (excitation 370 nm) is related to the level of other AGEs [34]. All emission spectra were normalized to sample mass content.

Statistical analysis

The analysis of differences between samples from young adult and ageing animals, both freshly isolated (Y–C vs. A–C) and ribosylated (Y–R vs. A–R), was performed using Student's t test. Effect of in vitro glycation was estimated in the terms of paired t test for related samples from the same animal incubated in buffer and in ribose. Statistical tests were performed using Statistica 10 (StatSoft Inc., USA). The 5% level of significance was applied for all tests.

Results

Results of DSC measurements for samples from young adult (Y–C) and ageing (A–C) rabbits, and a significance of differences between Y–C and A–C groups are presented in Table 1. The effect of ageing was tissue dependent. The temperature of denaturation did not change in the ageing cornea in respect of the young adult ones, while in meniscus and in tendon the temperature in the older group was significantly higher than in the younger one. Enthalpy of denaturation (ΔH_d) was significantly affected by ageing both in cornea and in tendon, but not in meniscus. However, in meniscus the half-width of the peak ($\Delta T_{1/2}$) in the ageing group was lower than in the young adult one.

Table 1 Parameters of thermal denaturation of cornea, meniscus and Achilles tendon in young adult (Y–C) and ageing (A–C) rabbits

	T_m/°C	ΔH_d/J g^{-1}	$\Delta T_{1/2}$/°C
Cornea			
Y–C	63.4 (0.93)	26.7 (2.9)	5.3 (0.7)
A–C	63.2 (0.73)	19.5 (1.6)	4.5 (0.7)
p value	n.s.	< 0.0001	n.s.
Meniscus			
Y–C	66.5 (0.4)	13.4 (1.2)	5.7 (0.6)
A–C	67.5 (0.5)	13.2 (1.0)	5.1 (1.1)
p value	0.0019	n.s.	n.s.
Achilles tendon			
Y–C	63.0 (0.31)	51.9 (2.3)	3.9 (0.4)
A–C	63.6 (0.27)	45.6 (1.7)	4.0 (0.8)
p value	0.0158	0.0003	n.s.

Mean values (standard deviation) are listed; $n = 6$ in each group

p values for significance of differences between Y–C and A–C calculated from Student's t test

Results of in vitro glycation for both young adult and ageing groups are presented in Tables 2–4, for cornea, meniscus and Achilles tendon, respectively. Parameters of collagen denaturation were influenced by ribose incubation, both in the young adult (Y-) and in the ageing groups (A-). In cornea (Table 2) and in Achilles tendon (Table 4), denaturation temperature increased, while enthalpy and peak half-width decreased, though the decrease in peak width in ageing cornea was not significant. Effect of ribation in meniscus (Table 3) was manifested as a significant increase in the denaturation temperature without any impact on enthalpy, both in the young and in the ageing group. The decrease in peak half-width was not significant. In ribosylated tissues, the thermal parameters presented in Tables 2–4 were not significantly different between the young adult (Y) and the ageing (A) groups.

However, significant effects of the incubation in buffer without ribose were observed in corneal samples both in the young adult (Y–B vs. Y–C) and in the ageing (A–B vs. A–C) ones. Incubation in the buffer lowered temperature of denaturation in the younger group ($p = 0.0256$) and increased the enthalpy in both age groups (Y: $p = 0.0130$; A: $p = 0.0048$) which could result from swelling of the cornea.

In Fig. 2, fluorescence spectra obtained for papain-digested tissues from the freshly isolated young adult (Y–C) and the ageing (A–C) groups are presented. In corneal samples, both Y–C and A–C, fluorescence after excitation with 335 nm and with 370 nm was residual. In meniscus, a noticeable fluorescence was recorded in both the Y–C and the A–C group, with maximum approximately 2–3 times more intensive in the ageing than in the young group for both bands. In tendon, a noticeable increase in fluorescence in both bands was recorded in the ageing group in respect of the younger one (about 5 times higher for 335 nm excitation and 3 times larger for 370 nm excitation).

Table 2 Parameters of thermal denaturation of cornea from young adult (Y-) and ageing (A-) rabbits incubated in ribose solution (-R) and in buffer (-B)

Cornea	$T_m/^oC$	$\Delta H_d/J\ g^{-1}$	$\Delta T_{1/2}/^oC$
Y–B	61.9 (1,0)	32.5 (3.7)	4.0 (0.4)
Y–R	66.5 (2.1)	22.7 (4.3)	2.9 (0.3)
p value	0.0011	0.0038	0.0152
A–B	61.5 (1.8)	26.0 (4.0)	3.6 (0.5)
A–R	68.3 (1.0)	21.2 (2.7)	3.1 (0.6)
p value	< 0.0001	0.0139	n.s.

Mean values (standard deviation) are listed; $n = 6$ in each group

p values for significance of differences between -B and -R samples calculated from paired t test

Table 3 Parameters of thermal denaturation of meniscus from young adult (Y-) and ageing (A-) rabbits incubated in ribose solution (-R) and in buffer (-B)

Meniscus	$T_m/^oC$	$\Delta H_d/J\ g^{-1}$	$\Delta T_{1/2}/^oC$
Y–B	65.8 (0.7)	14.7 (2.3)	5.2 (0.8)
Y–R	71.9 (2.8)	15.6 (3.9)	3.9 (0.9)
p value	0.0086	n.s.	n.s.
A–B	66.7 (0.9)	14.9 (1.4)	4.9 (0.8)
A–R	71.9 (0.9)	14.6 (2.2)	4.2 (0.8)
p value	0.0020	n.s.	n.s.

Mean values (standard deviation) are listed; $n = 6$ in each group

p values for significance of differences between -B and -R samples calculated from paired t test

Table 4 Parameters of thermal denaturation of Achilles tendon from young adult (Y-) and ageing (A-) rabbits incubated in ribose solution (-R) and in buffer (-B)

Achilles tendon	$T_m/^oC$	$\Delta H_d/J\ g^{-1}$	$\Delta T_{1/2}/^oC$
Y–B	63.0 (1.3)	45.2 (2.3)	3.6 (0.3)
Y–R	67.1 (1.9)	38.1 (3.8)	2.6 (0.4)
p value	0.0028	0.0014	0.0013
A–B	63.1 (1.0)	43.4 (2.0)	3.8 (0.4)
A–R	66.5 (0.9)	41.2 (1.5)	2.7 (0.4)
p value	0.0003	0.0062	0.0078

Mean values (standard deviation) are listed; $n = 6$ in each group

p values for significance of differences between -B and -R samples calculated from paired t test

Spectra of incubated samples are presented in Figs. 3 and 4. In all tissues, an increase in fluorescence intensity in ribose incubated samples as compared to buffer incubated ones was shown both in the young adult and in the ageing group. The most intensive increase in fluorescence after incubation in ribose was found in corneas and tendons from the younger group.

Discussion

The study was an attempt to compare ageing-related and in vitro glycation-induced changes of extracellular matrix collagen in cornea, meniscus and Achilles tendon from young adult and from ageing rabbits. The main macromolecule of extracellular matrix in these tissues is type I collagen, even though the other components and the structure of the matrix are different [35–37]. Collagen in tissues was frequently characterized in the terms of thermal denaturation parameters [24–32], and in particular, differences in temperature of denaturation were used as an
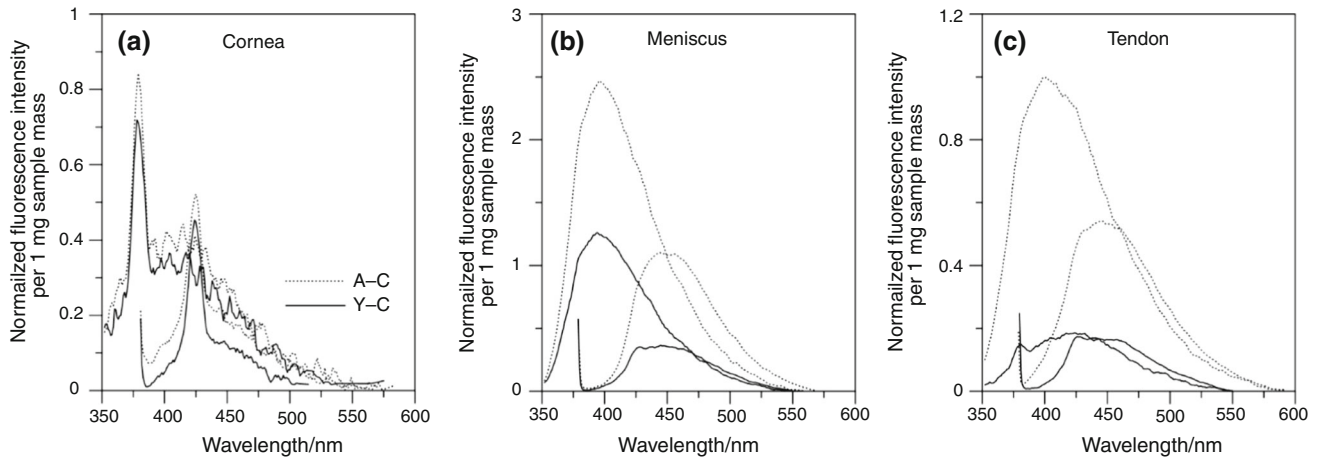
Fig. 2 Fluorescence spectra of papain-digested tissues from freshly isolated young adult (Y–C) and ageing (A–C) rabbits
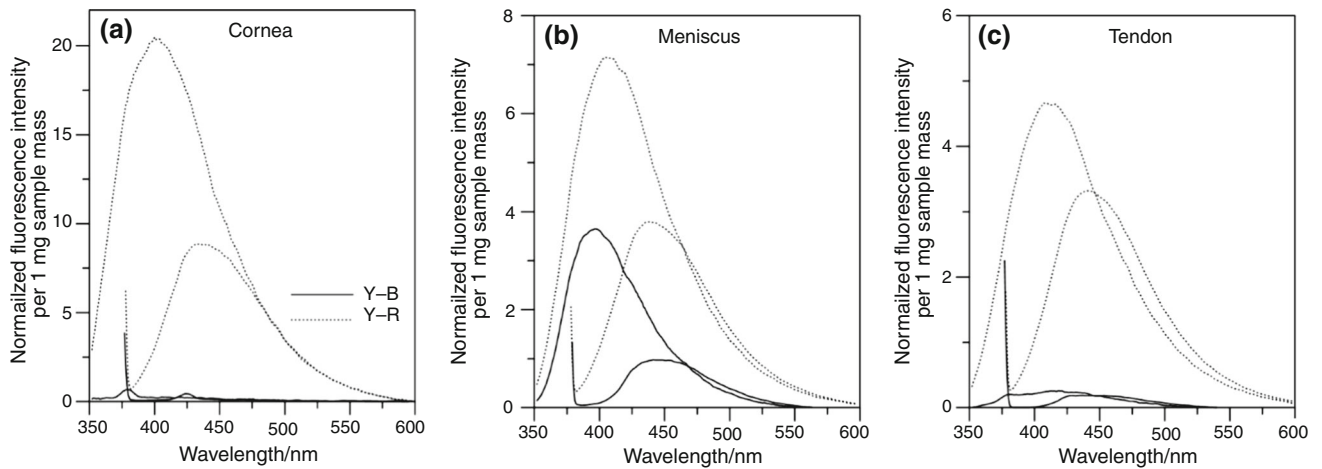

Fig. 3 Fluorescence spectra of papain-digested tissues from young adult rabbits after 2 weeks of incubation in buffer (Y–B) and in buffer with ribose (Y–R)
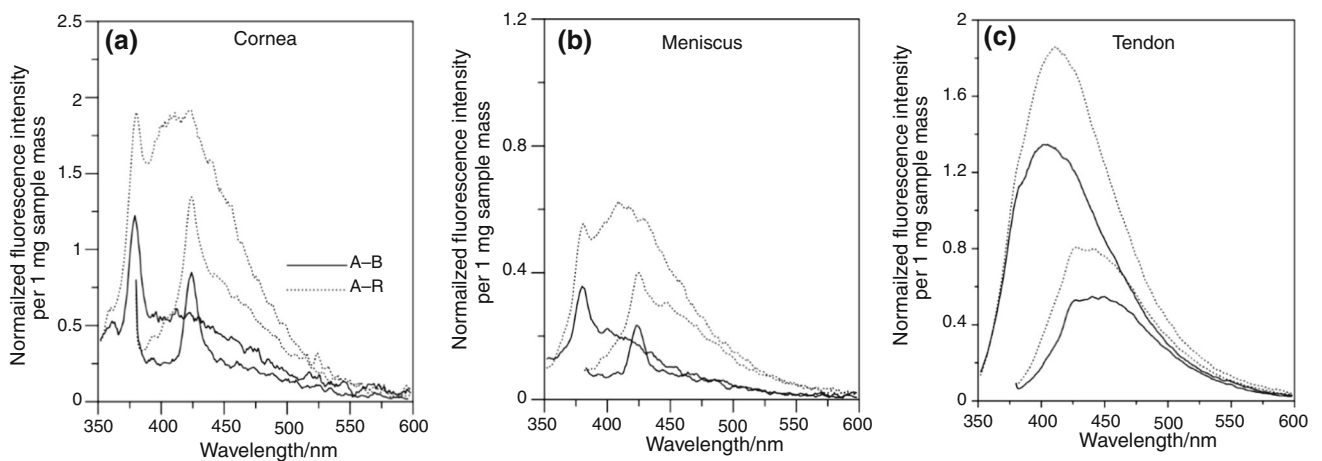

Fig. 4 Fluorescence spectra of papain-digested tissues from ageing rabbits after 2 weeks of incubation in buffer (A–B) and in buffer with ribose (A–R)

indicator of differences in collagen crosslinking on the basis of numerous reports from previous experiments [17, 18, 20, 21, 25, 27, 38].

The temperature of denaturation in corneal samples was not different between young mature (Y–C) and ageing (A–C) groups (Table 1). Accounting for the fact that after maturation of the tissue the only possible change in crosslinking profile of collagen can be caused by glycation [2, 3], a similarly low level of advanced glycation end products (AGEs) in both age groups could be inferred. That conclusion is confirmed by fluorescence intensity which was very low in samples from both young mature and ageing rabbits (Fig. 2a). The lack of pentosidine was reported also in a recent study on crosslinks in adult human corneas [39]. In other studies, no immunoreactivity to different types of AGEs was detected in normal human corneas in adults of different age [40] and in healthy corneas in monkeys [41]. As concerns thermal denaturation of cornea, very little literature exists [27, 39] and the only one reporting age-related changes [39] shows rather a slight decrease in denaturation temperature without any signs of age-related corneal crosslinking. The lack of glycation evidence in naturally ageing corneal samples can result from a relatively short half-life of type I collagen in cornea [37] or could be a sign of low susceptibility to AGEs formation under normal conditions.

A significant increase in denaturation temperature was found in ageing meniscus and Achilles tendon as compared to young adult ones (Table 1). Accounting for the fact that in both age groups the process of enzymatic crosslinks formation should be completed, the presence of non-enzymatic glycation products in the older group (A–C) can be presumed. Moreover, fluorescence intensity in ageing meniscus and ageing Achilles tendon (Fig. 2b, c) suggests much higher levels of pentosidine and other fluorescent AGEs than in the young adult tissues. It was found previously that pentosidine was positively related to age in human Achilles tendon [42–44] and in meniscus an exponential increase in pentosidine with age was stated [45]. As concerns calorimetric studies, an age-related increase in denaturation temperature in tendon was previously reported [21, 46], but there are no other reports on thermal denaturation of meniscus in relation to ageing.

Enthalpy of denaturation results from a combination of heat needed to disrupt the hydrogen bonds responsible for the secondary structure of collagen and energy to break up hydrophobic interactions of side-chains exposed to water during helix–coil transition [47]. So, the value of enthalpy arises from an intrinsic composition of the molecules, but is influenced by the presence of water surrounding the molecules in fibres. A significant decrease in enthalpy was shown in dehydrated collagen fibres from different tissues [19, 23, 24]. Even in a fully hydrated environment, like in our experiment, water can be pushed out from the fibres by the crosslinking-induced closer alignment [22, 23]. However, as long as the amount of water is bound to the molecule and water in a direct contact with the molecule in the fibre is not reduced, crosslinking-related dehydration of fibres should not affect the enthalpy of denaturation [22]. In the present study, a significant decrease in enthalpy was shown in fully hydrated samples of ageing Achilles tendon, but not in meniscus (Table 1), even though in the both tissues an increase in crosslinking was found. Thus, it is presumable that ageing-related crosslinks pushed out the water close to collagen fibrils in tendon, but not in meniscus. In all soft tissues, fibrils are embedded in highly hydrophilic proteoglycan-rich matrix. However, in tendon mid-substance small proteoglycans—decorin and biglycan—which decorate the fibrils at discrete binding sites are the most abundant [48, 49], while in the adult menisci the proteoglycan matrix contains also large, highly hydrated aggrecan aggregates [50].

In the ageing cornea, enthalpy of denaturation decreased significantly, even though no evidence of glycation crosslinks was found (Table 1). However, in the ageing cornea a closer alignment of collagen fibrils can be presumed. A perfectly regular structure of corneal stroma is formed from fine lamellae composed of narrow, uniform diameter collagen fibrils and the lamellae are separated by layers of hydrogel composed of small proteoglycan [37, 51]. It was shown that the corneal collagen interfibrillar spacing decreased with age, and this may be related to changes in the matrix proteoglycan composition [52, 53].

I should be stressed that ΔH measured in our study is expressed in relation to mass of the tissue sample, but not to mass of collagen, so any change in amount of collagen in the tissue would influence the enthalpy value even if no other changes occur. However, during ageing the collagen content in the tissues rises by a few per cent [49–51], so the amount of collagen cannot be a reason of enthalpy decrease stated in cornea and tendon samples.

Age-related changes in tissues were characterized also in the terms of the half-width of the denaturation endotherm ($\Delta T_{1/2}$). The half-width of the peak is an index of the cooperative nature of the transition of the molecules from native to denatured state. Peak width will decrease due to a better organization and homogeneity of the helices [16, 20, 23, 47, 54]. A broader denaturation peak should be observed in tissues where type I collagen is accompanied by other collagens populations that have different thermal stabilities [23, 54]. In general, broader denaturation peaks were found in cornea and meniscus than in tendon (Fig. 1, Tables 1–4), which can be attributed to differences in composition and organization of tissue matrix. In adult cornea, only 50–55% of collagens makes type I collagen [37, 52, 53], while in tendon 90% is type I [35, 49]. Type I

makes almost 90% of collagens also in meniscus [36, 50]. However, the cooperativity of collagen denaturation in meniscus can be lowered by a multidirectional and complex layout of fibres as well as by the presence of type II collagen.

Two weeks of incubation in ribose solution resulted in a significant increase in denaturation temperature as compared to the buffer incubated samples in all tissues in both age groups, which signals a considerably higher level of collagen crosslinking in glycated tissues (Tables 2–4). The effect of ribose was highly significant even though the scatter of results, expressed in terms of standard deviation, was larger than in the non-incubated, naturally ageing samples. A better organization of the collagen helices and/or homogeneity of fibres in ribosylated samples can be presumed also from the narrower denaturation endotherms, though the change of $\Delta T_{1/2}$ was insignificant in meniscus and in young adult cornea (Tables 2–4). Stabilizing effects of glycation were also shown in other DSC studies in tendon [20, 303], skin [20, 55], lens capsules [56] and bone [25].

One of limitations of the present study is the glycation procedure and an impact of the buffer itself on corneal samples. Cornea is particularly prone to swelling. Hydrophilic glycosaminoglycans surrounding the collagen fibrils attract water from buffer; the fibrils move apart and redistribute [37]. However, the swelling process did not eliminate the impact of ribose. Stabilizing effects of ribosylation on corneal samples were significant in terms of all analysed parameters of denaturation (Table 2).

Higher levels of pentosidine and other fluorescent AGEs in all groups of the ribosylated samples were revealed also in the level of fluorescence. In general, the increase in fluorescence intensity in the young adult tissues (Fig. 3) was larger than in the ageing ones (Fig. 4). A considerably large increase in fluorescent AGEs was found in young adult cornea. Accounting for the relatively short half-life of type I collagen in cornea [37], this increase can result from a larger amount of newly synthesized collagen molecules with a greater number of free lysine residues than in the tendon and meniscus. However, in the ageing cornea the ribosylation-related increase in fluorescence was several times lower than in the young adult one. On the other hand, the weakest reaction to incubation in ribose was found in meniscus, both young adult and ageing (Figs. 3b and 4b). The level of enzymatic crosslinking of collagen in adult meniscus is naturally higher than in other tissues [50], and thus the number of possible glycation sites during incubation was fewer.

Although the increase in fluorescence in ribosylated samples was tissue and age dependent, the increase in denaturation temperature was similar in all tissues in both age groups and did not correlate with the increase in fluorescence. This lack of correlation between the gain of fluorescent AGEs and temperature change was noticeable also including naturally ageing tissues. In meniscus, the increase in fluorescence was very similar for naturally ageing and ribosylated samples, but the temperature of denaturation rose by 1 °C in naturally ageing meniscus while the temperature increase in the young adult and the ageing ones incubated in ribose was 6.1 and 5.2 °C, respectively. In naturally ageing tendon, more than threefold increase of fluorescence was accompanied by 0.6 °C increase of denaturation temperature, while in the older group of ribosylated tendons, about 40% gain of fluorescent AGEs resulted in 3.4 °C of temperature increase.

These results imply that stabilizing effects of glycation depend not only on the amount of AGEs but also on their type and/or location. In vitro glycation in high concentration of ribose resulted most likely in crosslinks between helical parts of collagen molecules which influences the stability much more than non-helical crosslinks. Consequently, it seems that experiments with high concentration of sugar are not the best model for natural glycation processes, despite the advantage of a short incubation time.

Conclusions

Parameters of thermal denaturation of collagen in rabbit cornea, meniscus and tendon are tissue and age dependent and can be explained in terms of composition and structural differences. Amount of fluorescent AGEs in the tissues extracts is not the only determinant of increasing thermal stability of collagen, both in naturally ageing and in vitro glycated tissues.

Compliance with ethical standards

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