Review article

The role of collagen crosslinks in ageing and diabetes - the good, the bad, and the ugly

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Summary

The non-enzymatic reaction of proteins with glucose (glycation) is a topic of rapidly growing importance in human health and medicine. There is increasing evidence that this reaction plays a central role in ageing and disease of connective tissues. Of particular interest are changes in type-I collagens, long-lived proteins that form the mechanical backbone of connective tissues in nearly every human organ. Despite considerable correlative evidence relating extracellular matrix (ECM) glycation to disease, little is known of how ECM modification by glucose impacts matrix mechanics and damage, cell-matrix interactions, and matrix turnover during aging. More daunting is to understand how these factors interact to cumulatively affect local repair of matrix damage, progression of tissue disease, or systemic health and longevity. This focused review will summarize what is currently known regarding collagen glycation as a potential driver of connective tissue disease. We concentrate attention on tendon as an affected connective tissue with large clinical relevance, and as a tissue that can serve as a useful model tissue for investigation into glycation as a potentially critical player in tissue fibrosis related to ageing and diabetes.

KEY WORDS: collagen, advanced glycation end-products, crosslinks, tendon mechanics, diabetes, ageing.

Setting the stage: central functional roles of collagen

The term *collagen* comes from the Greek word κολλα (*kolla,* meaning "glue"), due to the use of animal skin and collagen-rich tissues a glue source[1]. In a broader sense, collagen is in fact the "glue" of our body, holding it together by providing elasticity and strength to most tissues where mechanical function is essential, such as skin, cartilage, tendons and bones [2,3].

The collagen family of proteins is the most abundant in the human body – representing a basic building block within nearly every tissue and organ. Collagen structures form largely by cell-mediated self-assembly of small collagen molecules (300 nm in length; circumscribable with an approximate 1.5 nm diameter)[4]. During the process of collagen self-assembly, various types of inter-molecular crosslinks stabilize the helical supramolecular structures that form. Collagen crosslinks can be conceptually classed as either enzymatic or non-enzymatic, with enzymatic crosslinking representing an essential step in the development and repair of collagen connective tissues. Whether in the early stages of embryonic tendon development or the late stages of connective tissue disease, collagen crosslinks play a key role in tissue mechanics, cell signaling, matrix damage accumulation, and tissue repair.

Cell-matrix interactions involving collagen include a wide range of classical receptor-ligand mediated signaling pathways[5]. Nonetheless the main functional feature of most collagens (this review will focus on type-I collagen) is mechanical load bearing of tensile force.The mechanical function of any connective tissue results from often highly sophisticated architectural arrangement of collagen substructures, along with other elastic extracellular matrix proteins such as elastin, and water binding proteoglycans. Although soft connective tissues of the body are composed of nearly identical basic molecular building blocks, their varied arrangement makes possible an exquisite range of potential tissue mechanical properties. The cells that mediate the functional assembly of these building blocks do so according to their epigenetic pre-program as guided by the mechanical demands on the tissue.

Within any collagenous connective tissue, the functional building blocks that provide tensile strength and elasticity are called collagen "fibrils". The collagen fibril is a helically arranged supramolecular structure that can range in diameter from a few to several hundred nanometers, with lengths that can run on the or-

der of centimeters[6]. How collagen molecules are accrued into these structures (a process known as fibrillogenesis) relies on sequences of elegant intracellular and extracellular events that, while fascinating, are outside the scope of the present review. Current evidence suggests that the mature collagen fibrils resulting from fibrillogenesis are highly elastic structures – meaning that they mechanically load and unload in a mostly reversible fashion. To be able to reversibly load and unload, without damage, is the defining functional requirement of these protein superstructures. Collagen cross linking is a central enabler (and potential disabler) of this function.

The good: enzyme mediated collagen cros-slinking

The mechanical competence of individual type-I collagen fibrils heavily depends on the enzyme lysyl oxidase, which regulates the robust formation of stable inter-molecular collagen crosslinks during maturation[7]. The absence of these head to tail chemical bonds drastically diminishes collagen fibril strength and whole tissue function[8,9]. Lysyl oxidase specifically acts on lysine or hydroxylysine in the telopeptide region of the collagen molecule, and results in a divalent, immature crosslink with an opposing amino-acid in the triple-helical region[10]. These immature crosslinks later spontaneously convert into more stable trivalent crosslinks that increase collagen interconnectivity, fibril stability and whole tendon mechanical integrity (for excellent reviews)[7,11].
Simple biochemical correlations of native crosslink content with tendon mechanical properties are rather weak[12-15], reflecting the likely confounding influence of other dominant structural or compositional factors[16]. The essential functional role of crosslinking in collagen fibril stability and whole tissue integrity, however, is clearly demonstrated in the severely compromised connective tissues of animals subjected to dietary inhibition of lysyl oxidase, which results in collagen fibrils and tendons with reduced strength[8,9]. The importance of crosslinks to fibril integrity has been indicated theoretically[17] and demonstrated experimentally[9,18] by balancing molecular slip and stretch under load.
The importance of crosslinking in preventing molecular slippage and resultant fibrillar damage can also be inferred from the decreased thermal stability of tendons that is known to take place after sub-maximal tissue overload[19]. Given that lysyl oxidase mediated crosslinks are so essential to the proper development of fibril structure and mechanical integrity, these are perhaps the best-characterized collagen crosslinkers.

The bad: advanced glycation endproduct crosslinking

While enzyme driven crosslinking plateaus at maturation, connective tissue stiffness has been shown to further increase with age and diabetes[20-26]. This tissue stiffening has been associated with non-enzymatic, oxidative reactions between glucose and collagen which lead to the formation of so-called advanced glycation end-products (AGEs)[27,28]. AGE accumulation is particularly high in long-lived proteins, such as collagen. Indeed, collagen half-life varies between tissues but remains generally large, from 1-2 years for bone collagen to about 10 years for type I in skin[29]. The low biological turnover of collagen makes it therefore susceptible to interaction with metabolites, primarily glucose. Aside from protein longevity, another factor that influences the formation of AGEs is the glucose level in the blood stream. Hyperglycemia related to diabetes is suspected to strongly predispose tissues of these patients to accumulation of AGEs[30,31].
The glycation reaction initiates with the formation of a reversible Schiff base between a carbohydrate – typically glucose – and a protein amino group (e.g., a collagen lysine side-chain) (Fig. 1). The unstable Schiff base becomes a stable intermediate keto amine, often designated asa so-called Amadori product. Afterwards, a complex series of reactions (over the course of months or years) lead to various meta-

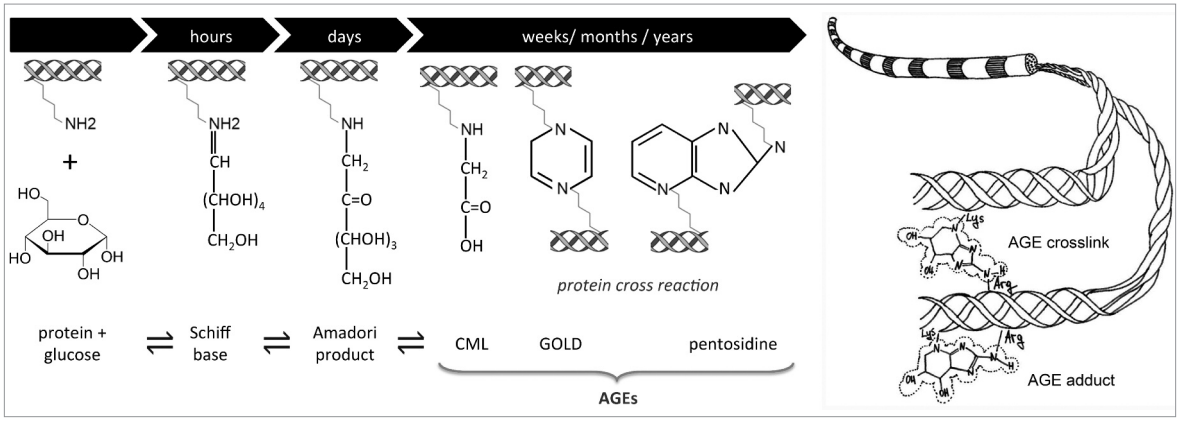

Figure 1. (Left) Schematic of the sequence of metabolic chemical reactions behind AGE formation (e.g. pentosidine)[72] and (Right) how such products may form adducts and/or crosslinks on collagen structures[39].

bolic by-products of glycolysis including the products glyoxal, methyl glyoxal (MGO) and 3-deoxyglucosone, all of which can interact with extracellular proteins to form AGEs[32]. Some AGEs can bridge between the free amino groups of neigh boring proteins to form inter-molecular crosslinks, while others known as 'adducts' affect only a single protein[33]. Among the different AGEs, the most abundant in collagen tissues has been recently found to be glucosepane, a lysine-arginine crosslink[34,35].

So far, there is no direct experimental evidence linking AGEs with increases in collagen fibril stiffness, which in turn would cause increased stiffness at higher levels of tissue architecture. Although the mechanical effects of AGEs at the molecular and supramolecular levels are poorly understood, this link seems plausible and has been widely presumed to exist on the basis of the well documented correlation between AGE markers (pentosidine; auto-fluorescence) and increasing tissue stiffness[36].

The ugly: functional consequences of AGEs in connective tissue

Despite the recognized importance of AGEs in the development of age – and diabetes – related conditions, there are still several important open questions regarding their role in the onset and progression of connective tissue disease. These can be broadly divided into two functional classes, biological and biomechanical.

The biological aspect relates primarily to collagen-protein and collagen-cell interactions. Here, the formation of AGEs (adducts or crosslinks) on specific amino acids involved in intermolecular recognition could lead to the dramatic modification of the interaction of collagen with other molecules such as proteoglycans (PGs), enzymes (e.g., collagenase) and cell integrins. AGEs modify the collagen surface and are known to affect cell-matrix interactions in a manner leading to inhibited wound repair and exacerbated inflammation[37,38]. A recent modeling study[39] based on atomistic model of collagen[40] has shown that collagen amino acids that are most likely prone to form glucosepane crosslinks (due to their position and configuration) are found close to collagenase and cell integrin binding sites, as well as near interaction domain for heparin and keratansulphate. These findings resonate with experimental investigations showing that collagen glycation induces a reduced affinity for heparin and keratansulphate proteoglycans (but not for dermatansulphate and decorin) as well as reduced endothelial cell migration[41]. Protein glycation ultimately stimulate cellular production of reactive oxygen species, and the activation of inflammatory signaling cascades via AGE signaling receptors (RAGEs)[42].

On the other hand, nonenzymatic intermolecular crosslinking are believed to alter the biomechanics of collagenous tissue. Glucose reaction with the amino acid side-chains, and subsequent further reaction to form a crosslink with an adjacent collagen molecule, results in a modification of the physical properties of the collagen, but the detailed effects of AGEs on collagen mechanics at the different hierarchical scales are still poorly understood. While these intermolecular crosslinks have been tied to higher failure loads, stiffness, and denaturation temperatures[30,43], they are also associated with increased mechanical fragility of the tissue[44]. AGE crosslinks have also been implicated in reduced remodeling capacity, a concept that has been demonstrated *in vitro* as reduced sensitivity to collagenase[43,45,46].

How collagen crosslinks affect whole tendon function is complex, as indicated by an increased failure load of individual collagen fibers that paradoxically yields diminished tissue failure properties. The picture is further muddied by contradictory reports in the literature that have inconsistently correlated crosslink density to tissue stiffness[13,44,47-52]. In an attempt to eliminate potentially confounding effects of genotype, systemic alterations due to age or disease state, and lifestyle, some studies have investigated the effects of crosslinking by direct incubation of tendon with a range of sugars and/or aldehydes solutions, serving as valuable models for ageing and diabetes (Fig. 2). These studies have generally well-mimicked the structural changes of collagen fibrils that have been found *in vivo*, but these studies clearly associate AGE crosslinks to tissue stiffening and brittleness[46,53,54]. Such changes are potentially critical, since altered extracellular matrix mechanics will subsequently affect the mechanical stimuli that drive resident cell behavior and regulate cellular repair of matrix damage. It is more than feasible that age-related mechanical changes in the collagen matrix could thus play a role in loss of tissue homeostasis and ability to cope with the micro-damage that accumulates in everyday life[27,28].

Clinical experience suggests that aged and diabetic connective tissues appear stiffer to the touch than healthy tissues, although changes in stiffness cannot be explained by increased collagen content alone[22,25]. Aged and diabetic tissues are also accompanied by characteristic yellowing of the collagen matrix that accords with experimental evidence indicating age-related decreases in collagen solubility and heightened collagen resistance to protease breakdown. These phenomena have been causally linked to non-enzymatic glycation of proteins[55-58].

The final duel: toward AGE crosslink breaking therapies

Various approaches have been taken to prevent formation of AGEs (for an excellent review)[59]. For instance, a reduced alimentary glucose uptake has been shown to be beneficial, as have approaches seeking to breakdown or block intermediate molecular interactions. Further efforts have shown potential benefit in "protecting" amino acid residues by agents that competitively bind aldehydes. Complementing

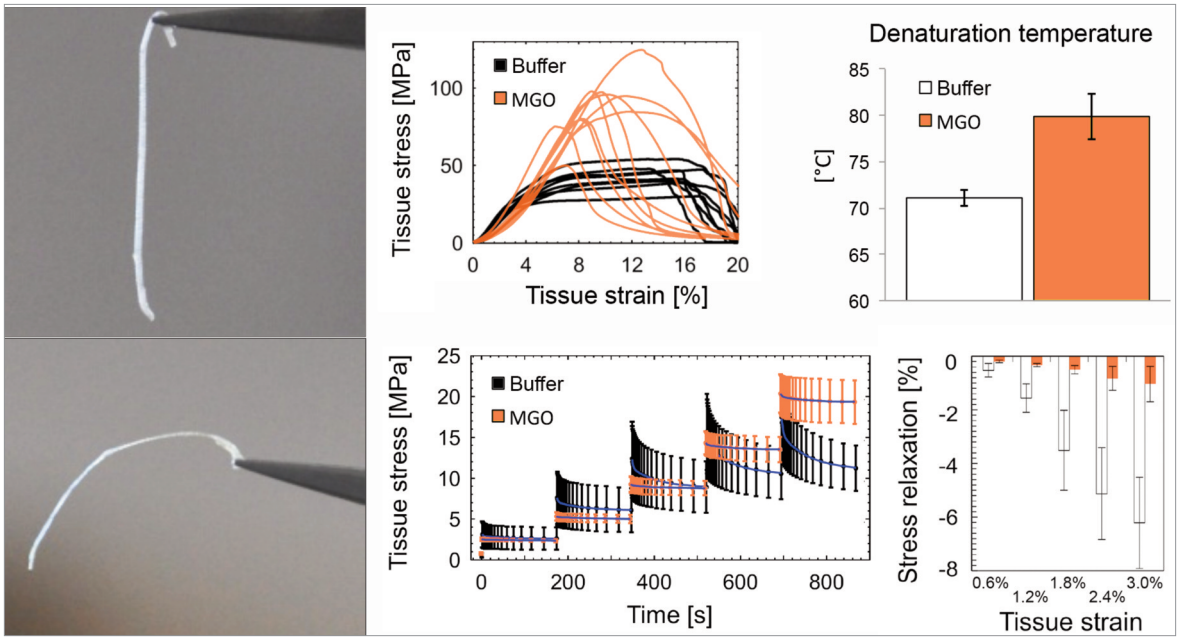

Figure 2. Crosslinking by AGEs induces various physical changes in type-I collagen dominated tissues. In the left-most panels, it can be seen that incubation of rat tail tendon fascicles in high concentrations of metabolite methylglyoxal (MGO) clearly affects tissue mechanics[72]. Closer investigation reveals that while tissue elasticity is only slightly affected, more dominant physical changes are observed in the viscoelastic properties of these tissues, their mode of tensile failure, and their resistance to thermal breakdown. All shown results has been obtained in research conducted ethically according to international standards[73].

these preventative approaches, some therapeutic approaches have sought to break existing AGE crosslinks. Contrary to the mentioned preventative approaches, crosslink breaking can reverse AGE crosslinking and its deleterious effects on tissue mechanics and matrix remodeling. Since AGE crosslinks in tendon are only secondary complications of diabetes, most anti-AGE work has been done in other tissues (such as skin and arteries). However, their potential effectiveness was first demonstrated using rat tail tendon[60]. At present, the most widely used crosslink breaker is alagebrium (ALT-711) which was shown able to reverse carotid artery stiffness in experimental models of diabetes[61]. However, it is not clear to what extent alagebrium efficacy in reducing diabetes related vascular and myocardial stiffness was due to the breaking of crosslinks. Such effects are also promoted by systemic effects of the drug on cytokine activity and/or oxidative stress reduction[62]. In any case, as far as we are aware there is no study testing the ability of crosslink breaking therapies to ameliorate the predisposition of tendon to mechanical damage, or promote "healthy" tissue remodeling at a repair site.

Another promising strategy for protein deglycation resides in the use of a family of deglycating enzymes[35,63,64], also called Amadoriases, Fructosyl Amino Acid Oxidases (FAODs) or Fructosyl Amine Oxidases (FAOX). These enzymes, found in fungi and bacteria, are able to cleave low molecular weight Amadori product (i.e., glycated amino acids) and yield the free amine, glucosone and hydrogen peroxide[65,66]. These enzymes have been categorized[67] into three classes depending on the substrate specificity: (i) active mostly on α-fructosyl amino acids (i.e., amino acids glycated on backbone amines), (ii) active mostly on ε-fructosyl amino acids (i.e., amino acids glycated on side-chains amine) and (iii) similar activity on either α- or ε-fructosyl amino acids. The most promising enzymes for protein deglycation are those active on amino acids side chains (ε-fructosamine), due to the larger number of potential glycation sites. However, despite the fact that from the first isolation of Amadoriase[68] over a dozen similar enzymes have been reported[67], none has shown significant activity on intact proteins, even after mutagenesis experiments[69,70]. One of the limiting factors in the development of deglycating enzymes with expanded substrate has been the uncertainty on their overall folding and conformation of active site. This limitation has been partly relieved by the finding of the crystal structure of Amadoriase II from *Aspergillus fumigatus*[71], possibly paving the way for the development of AGEs treatments.

A Summary

Collagen crosslinks strongly influence the mechanical and biological function of tendon tissue. While certain types of collagen crosslinks are essential to proper function, others can adversely affect tissue health. In this review, we attempted to distinguish crosslinks

that promote tissue strength, stiffness, and resistance to failure, from the non-enzymatic crosslinks that are associated with progressive collagen glycation in ageing and diabetes. Concerning the last class of crosslinks, we discussed possible therapeutic strategies to restore healthy tendon matrix mechanics.

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