

The Role of Nonenzymatic Glycation and Carbonyls in Collagen Cross-Linking for the Treatment of Keratoconus

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PURPOSE. Corneal cross-linking (CXL) is a treatment for keratoconus that eliminates the need for keratoplasty in most patients. However, its molecular mechanisms remain under study. Advanced glycation end products (AGEs) have been suggested by many studies as the causative strengthening agent during CXL, though no studies to date have directly tested this hypothesis.

METHODS. Corneas of young rabbits and sharks were pretreated with pyridoxal hydrochloride and copper ions before CXL. Two known inhibitors of AGE formation, aminoguanidine and rifampicin, were applied during CXL in the treatment solution. Tensile strength tests were conducted after these experiments to detect diminished or accentuated corneal stiffening after CXL. SDS-PAGE was performed on type I collagen cross-linked in the absence and presence of AGE inhibitors.

RESULTS. Pretreatment with pyridoxal hydrochloride resulted in significantly higher corneal stiffening after CXL. AGE inhibitors significantly diminished cross-linking as detected by both tensile strength measurements using whole corneas and gel electrophoresis of in vitro cross-linking of type I collagen in solution, in the presence and absence of the inhibitors. Rifampicin inhibited CXL more significantly than aminoguanidine in gel electrophoresis and tensile strength tests, confirming recent findings on its efficacy as an AGE inhibitor.

CONCLUSIONS. Data presented here suggest that CXL is carbonyl dependent and involves the formation of AGE cross-links. Six possible cross-linking mechanisms are discussed. (*Invest Ophthalmol Vis Sci.* 2011;52:6363–6369) DOI:10.1167/iops.11-7585

Corneal cross-linking (CXL) is an effective treatment to replace keratoplasty and hard contact lenses for treating patients in the early stages of keratoconus. During CXL, the deepithelialized cornea is typically “marinated” with riboflavin solution (RF) for 30 minutes and then irradiated with long-wavelength ultra-violet light (UVA) for 30 minutes as RF marination continues.¹ The UVA photosensitizes riboflavin, gener-

ating reactive oxygen species thought to be required for the formation of covalent cross-linking in the corneal extracellular matrix. These reactive oxygen species also cause the apoptosis of keratocytes in the anterior half of the corneal stroma.² Additionally, treatment with UVA is associated with some risk to other tissues within the eye.^{3–5} For this reason, it is desirable to develop protocols that increase the amount of cross-linking and yet allow a reduction of cytotoxic-UVA exposure.

Despite vast clinical knowledge of the applications and effects of CXL, the exact molecular mechanisms of cross-linking are mostly unknown.⁶ McCall et al.⁷ describe the many chemical interactions that are possible in CXL, pointing out that collagen fibrils were observed by Wollensak² to increase in diameter during CXL. Considering that it has been observed that an apparently similar increase in collagen fibril size in the corneal stroma occurs with such normal aging processes as glycation⁸ and the fact that the mean age of keratoconus onset is approximately 15 years and diminishes with age, it seems possible that age-related reactions, such as nonenzymatic glycation, may occur during CXL. It has also recently been identified that carbonyl groups and reactive oxygen species are essential to cross-linking, but the exact molecules involved remain unknown.⁷ Recent work indicates that CXL generates cross-links not only between collagen molecules but also between proteoglycan core proteins.⁹

Pyridoxal, the nonphosphorylated form of biologically active vitamin B₆, has been shown to nonenzymatically convert free primary amine residues to carbonyl residues by Schiff base formation between the carbonyl group of pyridoxal and a free amine residue in the presence of metal ions.¹⁰ Reacting the treated cornea with Brady’s reagent, which stains carbonyls red, can test for this chemical conversion of an amine to a carbonyl group. However, pyridoxal and its derivatives are also strong singlet-oxygen quenchers,¹¹ so their addition to the riboflavin solution would remove the necessary singlet oxygen and thus would inhibit UVA-catalyzed cross-linking. Nevertheless, if pretreatment with pyridoxal hydrochloride is followed by extensive rinsing and then CXL (thus removing such singlet-oxygen quenchers), a stronger cross-linking effect should be achieved than in achieved with CXL alone because many more carbonyl groups ought to be present and reactively available.

It is well documented in food science that cross-links slowly form between proteins and sugars attached to other proteins through Maillard reactions, also referred to as browning reactions or glycation, thus forming advanced glycation end products (AGEs).¹² AGEs form when reactive oxygen species chemically oxidize sugars, to spontaneously condense¹³ or form reactive metabolic intermediates, such as glyoxal.¹⁴ These reactive species then form Schiff bases with arginine and lysine residues in collagen fibrils¹⁵ and undergo a set of oxidations, rearrangements, and reductions to form cross-links in collagen.¹⁶ It also has been well documented that reactive oxygen

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species lead to the production of AGEs on specific lysine and hydroxylysine residues in type I collagen,¹⁷ although no studies to date have tested the possibility of this reaction in CXL.⁶ AGE cross-links cause the diameters of collagen fibrils in collagenous tissues to increase with aging¹⁸ and diabetes,¹⁹ both of which are associated with preventing the onset of keratoconus. Collagen fibril diameter is also seen to increase during CXL,² suggesting that AGE formation may be involved in CXL.

The corneal stroma contains abundant proteoglycan core proteins posttranslationally modified with the sulfated polysaccharide chains glycosaminoglycans (GAGs). These GAGs have carbonyl groups and sugars along their lengths that are in potentially reactive proximity to collagen fibrils.²⁰ These sugar-protein interfaces, which are essential for normal stromal structure,²¹ seem likely places for AGE cross-links to form. Even a small number of such cross-links between such large polymers as collagen fibrils and proteoglycans can be predicted to have disproportionately large effects on the tensile strengths of the corneal stroma in vivo.

Aminoguanidine (AG) and rifampicin (Rif) strongly inhibit the formation of AGEs,^{22,23} although Rif exhibits higher inhibitory potential than AG.²⁴ Neither of these AGE inhibitors is known to be capable of breaking preexisting AGE-derived cross-links.^{24,25} Rather, they inhibit the formation of new AGEs, possibly by reacting with the reactive carbonyl groups that AGE formation produces, thereby disabling the ability of carbonyl groups to react with amine residues.²⁶ Therefore, if the mechanism by which CXL causes cross-linking of corneas involves AGEs or reactive carbonyl groups, then the addition of an AGE inhibitor to the RF solution should significantly reduce the amount of cross-linking during CXL. Some of these hypotheses have been tested in the experiments described here.

MATERIALS AND METHODS

Tissue Acquisition and Storage

Whole rabbit eyes were received on dry ice from Pel-Freez Biologicals (Rogers, AR) and were stored in a -80°C freezer. Rabbit corneas were excised from still frozen rabbit eyes by first circumcising the sclera approximately 4 mm from the cornea and then carefully gripping the 4-mm margin of the sclera with jeweler's forceps to release the anterior half of the eye from the still frozen lens surface. Corneas were thawed in room temperature $1\times$ PBS, pH 7.4, for 2 minutes and subsequently deepithelialized by gentle scraping with a spatula over the anterior surface of the cornea. Corneas were then placed in a fresh solution of $1\times$ PBS immediately before treatment.

Whole eyes from dogfish sharks (*Squalus acanthias*) were obtained from Mount Desert Island Biological Laboratory (Salsbury Cove, ME). Corneas were shipped, stored, and prepared exactly as were the rabbit corneas, except 0.53 M NaCl, isotonic to seawater, was used in place of $1\times$ PBS.

Solution Preparation

Clinical riboflavin solutions were made by first dissolving in $1\times$ PBS (for rabbit corneas) or 0.53 M NaCl (for shark corneas) enough riboflavin-5'-phosphate (Fluka; Sigma-Aldrich, St. Louis, MO) to make a 0.1% wt/vol solution and, in the same solution, dissolving enough high-molecular weight dextran (Fisher Scientific, Pittsburgh, PA) to make a 20% wt/vol solution. The control solutions were made similarly to the process used for dextran, but they contained no riboflavin.

Pretreatment solutions for rabbit corneas were made by dissolving pyridoxal-hydrochloride (Sigma-Aldrich) in $1\times$ PBS (or in 0.53 M NaCl for sharks) to yield a 20-mM concentration, and copper sulfate (for catalysis) to yield a $1\text{-}\mu\text{M}$ concentration. This solution was then titrated to pH 7.4.

Aminoguanidine or Rif was dissolved in the previously described clinical riboflavin solution or control solution to yield 0.1% (wt/vol), depending on the designated treatment.

Pyridoxal Hydrochloride Pretreatment

For the pyridoxal pretreatment, freshly prepared corneas were individually placed in 50-mL centrifuge tubes containing 15 mL of 20 mM pyridoxal hydrochloride solution and were incubated gently on a rocker for 1 hour. After incubation in pyridoxal solution, the corneas were rinsed free of nonreacted pyridoxal (and also free of the pyridoxamine predicted to have been produced by transamination) by placing them on a rocker in 50-mL centrifuge tubes containing 45 mL $1\times$ PBS or 0.53 M NaCl, pH 7.4, for 15 minutes. This rinsing procedure was repeated to yield rinsing times of 0.5 hour, 1 hour, 2 hours, and 4 hours, so as to determine the effect of extensive rinsing on biochemical changes in the corneal stroma.

CXL Treatment and Tensile Strength Measurement

Corneas were draped on the top of ping-pong balls to maintain their native curvature during treatment. They were then dripped on from 2 cm above with the designated treatment solution at a rate of $10\ \mu\text{L}/\text{min}$ delivered by a syringe pump (model NE4000; New Era Pump Systems, Inc., Wantagh, NY), causing a drop to fall every 1.5 minutes. After 30 minutes, corneas were irradiated with ultraviolet light (365 nm, UVA, $3\ \text{mW}/\text{cm}^2$) at a distance of 5 cm from the clinical CXL irradiation apparatus (UV-X Radiation System for CXL; IROC Medical, Zurich, Switzerland), while dripping continued at the same rate. Regardless of whether the treatment involved UVA exposure, treatment solution was dripped onto all corneas for a total of 1 hour. After this treatment, corneas were quickly rinsed free of the viscous dextran solution in a Petri dish containing $1\times$ PBS or 0.53 M NaCl. Two-millimeter strips were cut with a scalpel from the center of the cornea, leaving sclera on each of the strips. Tensile strength tests were then performed as described in McCall et al.⁷ Briefly, these 2-mm corneal strips were attached at both ends to clamps. One of these clamps was attached to a force gauge, and the other was attached to an apparatus that moved away from the force gauge at a constant speed. The strengths reported were force gauge readings from corneal strips that were destroyed between the two clamps.

Staining with Brady's Reagent

Brady's reagent containing 2,4-dinitrophenylhydrazine (2,4-DNP) was prepared as described by McCall et al.⁷ Corneas underwent 1 of 2 treatments: treatment with $1\times$ PBS or 0.53 M NaCl and treatment with 20 mM pyridoxal in $1\times$ PBS or 0.53 M NaCl, in accordance with the pyridoxal pretreatment regimen. Then each was placed for 5 minutes in Brady's reagent with gentle rocking, rinsed in 10 mL of 100% methanol for 1 minute, placed into 1 N NaOH for 20 seconds, rinsed again for 1 minute in 10 mL methanol, which then was replaced with $1\times$ PBS or 0.53 M NaCl for 20 minutes, with gentle rocking. Digital photographs were taken immediately after this treatment.

SDS-PAGE

Sample Preparation. Soluble collagen type I ($3\ \mu\text{g}/\mu\text{L}$) from bovine skin was adjusted to pH 7.2. Type I collagen ($3\ \mu\text{L}$), pH 7.2, and $1\times$ PBS ($7\ \mu\text{L}$) were added to each of the treatment solutions ($10\ \mu\text{L}$). For the collagen control, $10\ \mu\text{L}$ of $1\times$ PBS was then added. With the other treatments, this $10\ \mu\text{L}$ of $1\times$ PBS contained twice the desired concentration of reagents, to yield a final volume of $20\ \mu\text{L}$ per treatment tube and with all reagents in desired final concentrations. Each of these tubes was then vortexed for 10 seconds and irradiated for 30 minutes at a distance of 5 cm with the corneal cross-linking radiation system (UV-X; IROC AG, Zurich, Switzerland) with the top of the solution exposed directly to the UVA. To ensure homogenous cross-linking, the tubes were vortexed for 10 seconds every 10 minutes during treatment.

Sample Analysis. For analysis of collagen cross-linking in solution, 5 μ L of 4 \times sample buffer (NuPAGE LDS; Invitrogen, Carlsbad, CA) and 2 μ L of 10 \times reducing agent (NuPAGE; Invitrogen) were added to each sample solution, heated at 70°C for 10 minutes, and loaded onto precast gels (8 cm \times 8 cm \times 1.5 mm; NuPAGE Novex Tris-Acetate Mini 3-8%; Invitrogen) and subjected to electrophoresis (120 V, 55 minutes) under reducing conditions. After electrophoresis, the gels were stained with 0.1% (wt/vol)

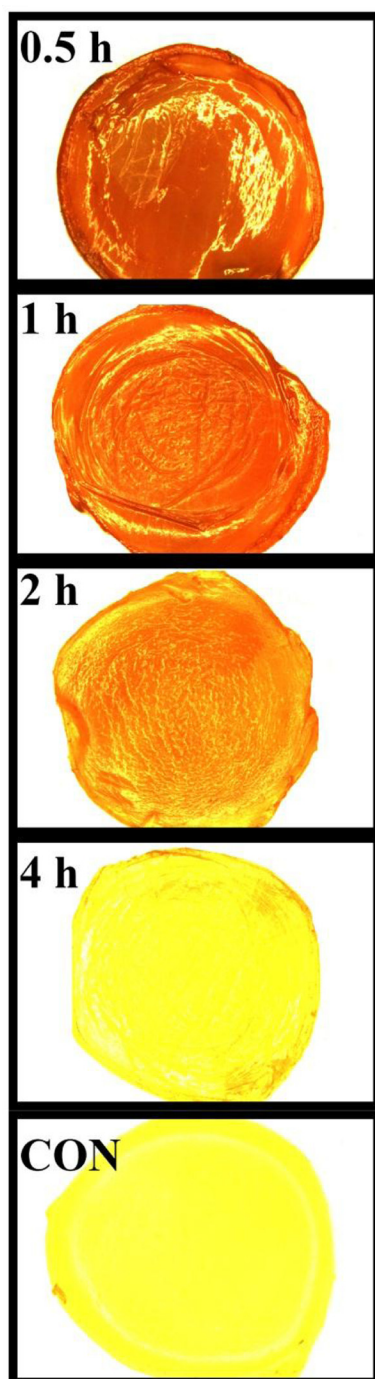


FIGURE 1. Results of histochemical staining for the presence of carbonyl groups with Brady's reagent containing 2,4-DNP in rabbit corneas. Rinsing time in 1 \times PBS is displayed in upper left corners of the boxes. A positive stain for carbonyl groups is the presence of red-violet color complexes in the tissue. Complete elution of bound pyridoxal was seen after 4 hours of rinsing. $n = 3$.

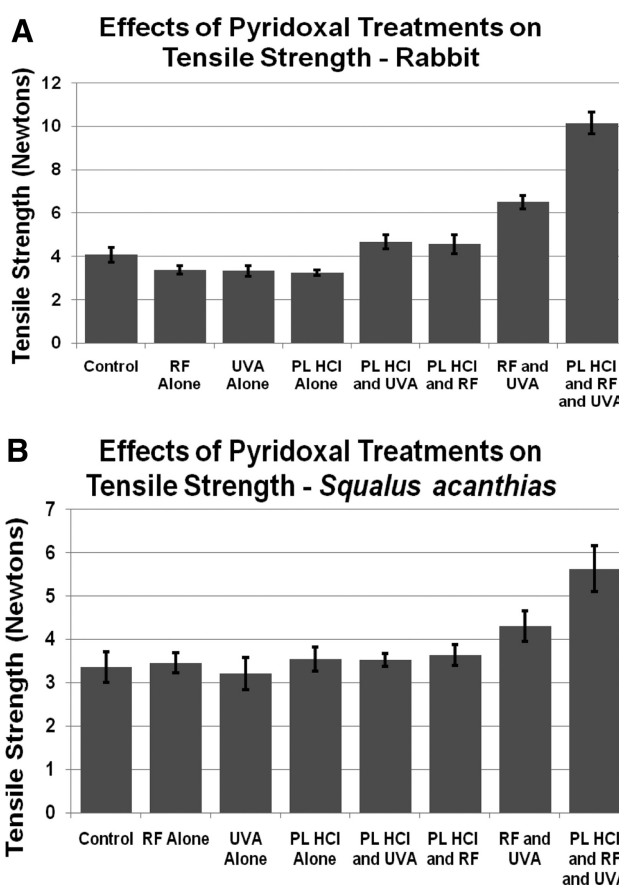


FIGURE 2. Treatments are described on the x -axes. Pyridoxal-hydrochloride (PL HCl) and pyridoxal phosphate (PLP) denote pretreatment with that chemical. Tensile strength of 20 mM PL HCl solution-pretreated and control rabbit corneas at pH 7.4. Pretreatment with PL HCl gave 156% increases in strength over CXL treatment in rabbits and 131% in sharks. Corneas were cut into 2-mm-wide strips after the final treatment step and were mounted in the apparatus for determining tissue strength. Controls were not statistically different; $n = 8$ corneal strips (4 corneas) per group. Error bars are expressed as SEM.

Coomassie Brilliant Blue R-250 overnight and subsequently destained in distilled water.

RESULTS

Pyridoxal hydrochloride solution initially appeared to oxidatively deaminate free amino groups to carbonyl groups on proteins in the deepithelialized cornea when rinsed for 0.5 hour or 1 hour (Fig. 1). However, after rinsing for 2 hours and 4 hours, staining by Brady's reagent diminished to control levels as bound pyridoxal was eluted from the corneal matrix.

Tests of the tensile strengths of pretreated and non-pretreated corneas were conducted using both rabbit and shark corneas (Fig. 2). Strengths of control corneas were all significantly lower than those of corneas treated with both RF and UVA. Pretreatment with pyridoxal hydrochloride solution before treatment with RF and UVA caused a significant increase (130% in sharks, 156% in rabbits) in strength over corneas treated with RF and UVA alone (4.31 ± 0.36 N compared with 5.63 ± 0.53 N in sharks; 6.51 ± 0.31 N compared with 10.16 ± 0.50 N in rabbits; $\alpha = 0.05$).

Tensile strength tests also were conducted to test whether the presence of the AGE inhibitors AG and Rif would cause a diminution of cross-linking during CXL treatment (Fig. 3). The

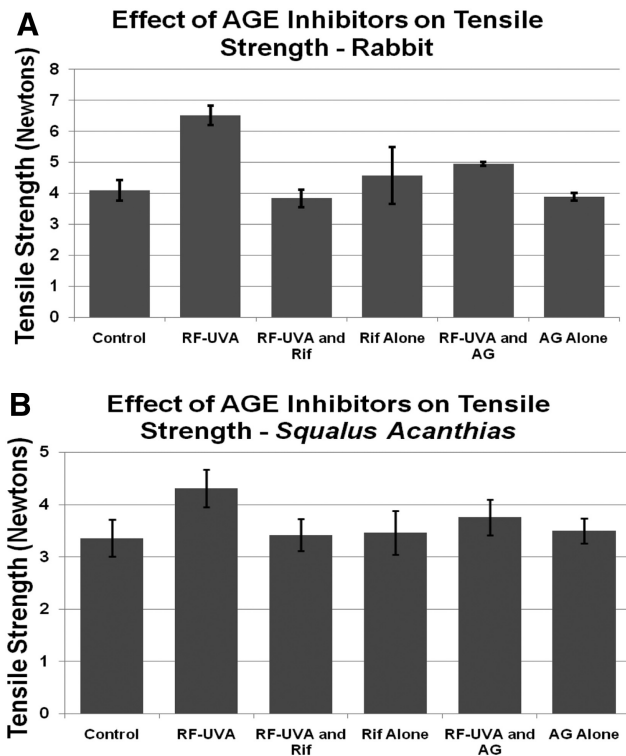


FIGURE 3. Tensile strengths of rabbit and shark corneas undergoing CXL in the presence of the AGE inhibitors Rif and AG. Treatments are described on the x-axis. Rifampicin shows greater ability to suppress cross-linking than AG in both sharks and rabbits; $n = 8$ corneal strips (4 corneas) per group. Error bars are expressed as SEM.

presence of the inhibitors alone showed no significant alteration of corneal tensile strength compared with controls. However, on the addition of Rif during CXL treatment, corneal strengths remained at control levels. The addition of AG during CXL caused a significant decrease in tensile strength compared with the addition of CXL alone but did not completely eliminate the strengthening effects of CXL.

Gel electrophoresis was used to determine the effects of these AGE inhibitors on in vitro CXL of soluble type I collagen (Fig. 4). In the absence of CXL, control collagen (lane 2) showed distinct native bands at 130, 250, and >250 kDa, corresponding to $\alpha 1/\alpha 2$ (monomer), β (dimer), and γ (trimer) chains, respectively. Collagen cross-linked by CXL (lane 3) alone showed almost complete disappearance of these bands (cross-linked collagen remained in the sample well as polymers too large to enter the gel). On the addition of AG during CXL (lane 4), instead of remaining cross-linked in the sample well, a significant proportion of the collagen was cross-linked only to the extent that it migrated as γ chains, and trace amounts remained non-cross-linked as $\alpha 1/\alpha 2$ and β bands, indicating an inhibition of cross-linking compared with that seen in the absence of AG (lane 3). The addition of 3 mM glucose to the AG-inclusive treatment (lane 5) neither increased nor decreased the pattern of inhibited cross-linking caused by AG. The addition of Rif, a more powerful inhibitor of cross-linking than AG, almost totally blocked cross-linking and generated a near-control state (lane 6), even in the simultaneous presence of 3 mM glucose (lane 7). As a control, the addition of 3 mM glucose to the collagen solution during CXL cross-linking (lane 8) caused only slight inhibition of collagen cross-linking compared with that seen in its absence (lane 3) as a variety of polymers approximating the molecular size of γ chains appeared, but

$\alpha 1/\alpha 2$ and β molecules were still totally cross-linked into higher molecular weight forms.

DISCUSSION

Results from the tensile strength tests after pretreatment with pyridoxal hydrochloride suggest that pyridoxal hydrochloride changes the biochemical properties of the corneal stroma in a way that facilitates cross-linking. Increasing the number of carbonyl groups in the stroma through oxidative deamination should increase the cross-linking effect of CXL because current evidence indicates that the process is carbonyl dependent.⁷ Alternatively, pyridoxal could covalently attach to amino groups, which can be excited by UV light to form reactive radical states.²⁷ These reactive radical states could then induce cross-links with other chemical groups in the stroma. The initial staining by 2,4-DNP-tests (Brady's reagent) after pretreatment with pyridoxal hydrochloride and a rinsing time of 0.5 hour or 1 hour indicate that oxidative deamination is occurring as staining is retained (Fig. 1). However, after 4 hours of rinsing time, it was clear from Brady staining that the biochemical change caused by pyridoxal hydrochloride was able to be reversed by further rinsing. This rinsing effect suggests that oxidative deamination is not responsible for the increased

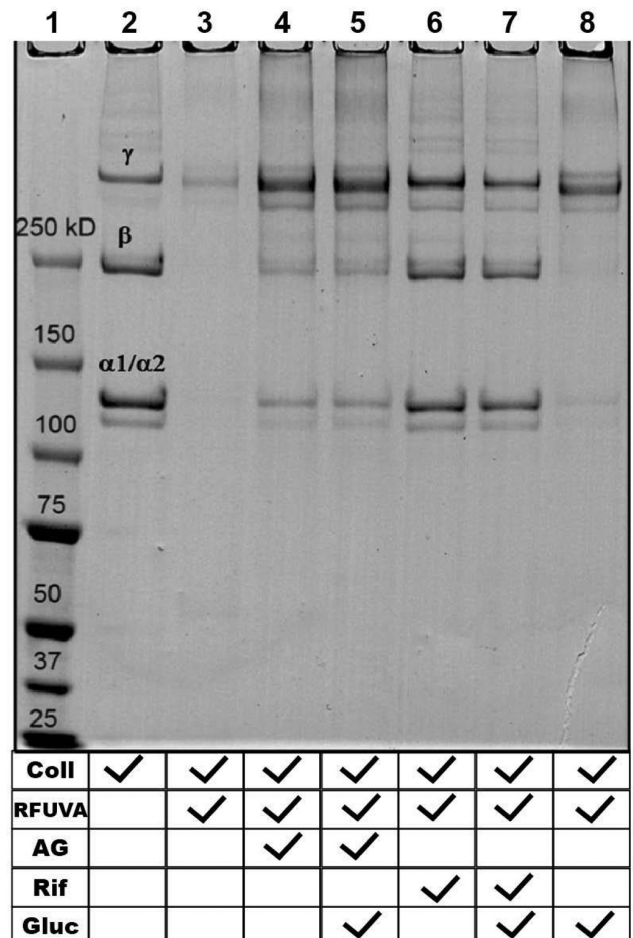


FIGURE 4. Electrophoretic analysis of AGE inhibitors. 9 μ g collagen/lane. Lane 1, molecular weight marker; lane 2, collagen alone; lane 3, collagen + CXL; lane 4, collagen + CXL + AG; lane 5, collagen + CXL + AG + 3 mM glucose; lane 6, collagen + CXL + Rif; lane 7, collagen + CXL + Rif + 3 mM glucose; lane 8, collagen + CXL + 3 mM glucose.

corneal stiffness on pretreatment with pyridoxal but, rather, that transiently bound pyridoxal is activated to reactive states by UV light. The aromatic ring of pyridoxal is activated analogously to tyrosine as a target for singlet oxygen, generating productive cross-links and more reactive species capable of displaying the observed increase in tissue tensile strength.^{28,29} Clinical use of a pyridoxal-based pretreatment on the cornea has not been reported previously for primary effects or potential side effects, but, because of the 2-hour pretreatment and the full immersion of the cornea in solution, it is unlikely that this protocol will have a direct clinical application. This chemical technique could be used in the field of collagen tissue

engineering as a way to selectively enhance cross-linking in certain areas of the engineered matrices.^{30,31}

Recent clinical studies of CXL already suggest that AGE formation might be responsible for the strengthening effect from CXL on the corneal stroma. First, persons with diabetes are less likely than those without diabetes to develop keratoconus.³² The high concentrations of blood glucose in diabetic patients causes increased AGE cross-links in many tissues of the body, including the cornea.³³ These cross-links could be providing structural integrity similar to that of postoperative CXL corneas, which would thereby reduce the likelihood of keratoconus onset. Second, a study investigating the smoking habits of keratoconus patients

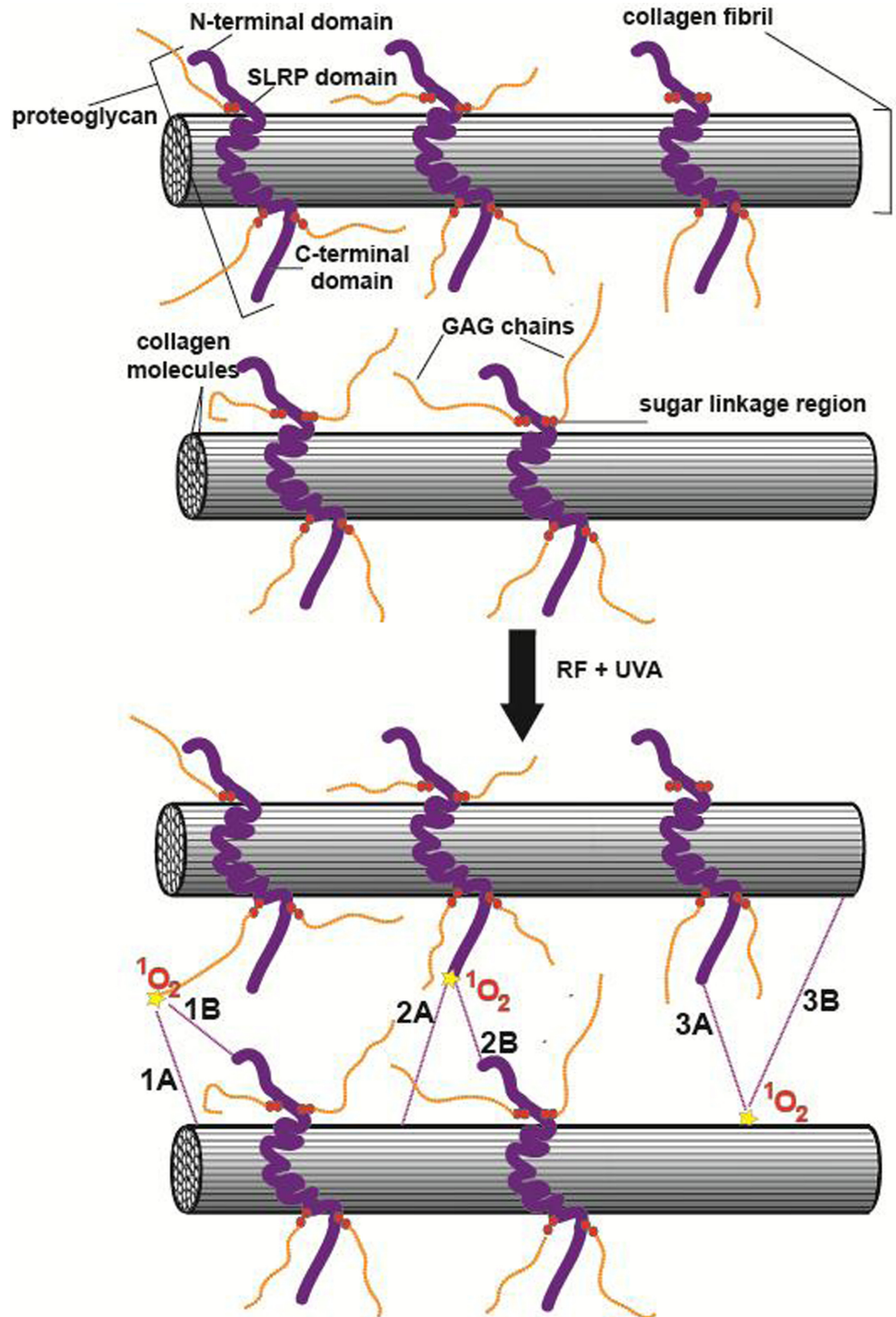


FIGURE 5. Diagram of proposed cross-linking mechanisms at the molecular level in the corneal stroma. *Top:* stromal structure before singlet-oxygen generation; *bottom:* six proposed chemical modifications to the stroma. *Star:* where singlet-oxygen reacts; *Dotted line:* possible places where modification could react. The six possible mechanisms are as follows: modification of sugar residue on GAG chain, which then reacts with a collagen molecule (1A) or a proteoglycan core protein (1B); modification of amino acid of a proteoglycan core protein, which then reacts with collagen (2A) or an adjacent proteoglycan core protein (2B); modification of an amino acid of a collagen molecule, which then reacts with a proteoglycan core protein (3A) or an adjacent collagen molecule (3B).

who underwent CXL showed lower incidence of keratoconus among smokers compared with non-smokers.³⁴ Cigarette smoke contains toxic substances that have been shown to increase the formation of AGEs in several tissues.³⁵

Furthermore, keratoconus tends to appear in patients early in life, before oxidative stress has produced significant numbers of AGE cross-links. These cross-links strengthen the cornea with age, making it more resistant to proteolytic degradation.³⁶ These AGE cross-links could protect the cornea from keratoconus in older subjects, which would explain why their induced formation during CXL inhibits the progression of keratoconus. One common factor linking diabetes, cigarette smoking, and aging is the formation of AGE cross-links. Another factor linking these three conditions is a reduction of corneal cortisol content.³⁷

Increased intrafibrillar volume and decreased interfibrillar spacing of corneal collagen fibrils have been documented in aging,¹⁸ diabetes,¹⁹ and CXL.² These same effects on collagen organization have been reproduced by inducing glycation in the rat tail tendon, suggesting that AGE cross-links are responsible for the increased intrafibrillar volume and decreased interfibrillar volume. It has been proposed that this occurs by the addition of other molecules to the interior domains of the collagen fibrils by AGE cross-linked glycosaminoglycans,³⁸ thus increasing the intrafibrillar volume.

The results shown here from the tensile strength tests and the gel electrophoresis are consistent with the formation of AGEs during CXL. Increased stiffening, as measured by tensile strength, was observed in CXL corneas, which is consistent with stiffening of the cornea resulting from glycation by age-related processes³⁶ and diabetes. AG and Rif showed different inhibitory strengths in both the tensile strength and the gel electrophoresis tests; these data are consistent with a recent findings showing that Rif is a stronger AGE inhibitor than AG.²⁴ Free glucose in the solution with collagen type I (Fig. 4, lane 8) inhibited the formation of the highest molecular weight polymers (remaining in the sample well) but allowed the formation of polymers of approximately γ -chain size yet did not inhibit the cross-linking-induced disappearance of the $\alpha 1/\alpha 2$ and β chains caused by CXL, suggesting that free glucose in the corneal stroma might slightly inhibit CXL compared with the pattern of cross-linking in the absence of free glucose (Fig. 4, lane 3). CXL may involve any of six possible reactions catalyzed by the presence of reactive oxygen species among collagen, proteoglycan core proteins, and glycosaminoglycan chains (Fig. 5), each of which can now be assessed. Zhang et al.⁹ demonstrates the role of both collagen and proteoglycan core proteins in CXL (mechanisms 1B, 2A, 2B, and 3A; Fig. 5). However, because of the short half-lives of glycosaminoglycan chains in the corneal stroma and results indicating that they are unreactive with collagen and proteoglycans during CXL in vitro,⁹ it is unlikely that these groups are involved (mechanisms 1A, 1B; Fig. 5).

In conclusion, the data presented here provide additional evidence implicating carbonyl groups in CXL and the additional efficacy of pretreatment with pyridoxal on the cross-linking procedure. The data also suggest a possible practical technique to increase the strengthening of CXL or to reduce exposure to cytotoxic UVA by pretreating with pyridoxal and copper ion, though this hypothesis has not been tested. One negative side effect of adding copper ions is that they could be involved in Fenton reactions that are deleterious to the strengthening effects of CXL. Finally, AGE formation could play a major role in the strengthening effect of CXL, verified by CXL-producing effects on corneal structure and rigidity similar to glycation in aging, diabetes, and cigarette smoking.

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