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INVITED REVIEW

Cross-Linked Hyaluronic Acid as Tear Film Substitute

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Abstract

Purpose: The aim of this review is to clarify the role of cross-linked Hyaluronic acid (HA) molecule as a tear supplement and to define its possible applications in dry eye disease.
Methods: Current Literature about HA and its cross-linked derivatives has been examined.
Results: HA is superior in increasing the viscosity and stability of the tear film compared with other tear supplements such as polyvinyl alcohol, hydroxypropyl methylcellulose, carboximethyl cellulose and polyethylene glycol. Moreover, HA can be modified in different ways to improve its properties such as molecular weight, viscosity, and hydrophobicity to adapt the new artificial molecule to different aims.
Conclusions: The current pharmacological trend is to improve the properties of HA by cross-linking parts of the molecule to achieve better bioavailability and resistence to degradation. In dry eye disease, cross-linked HA as tear supplement seems to provide better ocular comfort than linear HA and is therefore subjected to growing interest and diffusion.

Keywords: hyaluronic acid, cross-linked hyaluronic acid, dry eye disease

Introduction

Hyaluronic acid (HA; D-glucuronic acid [1-β-3] N-acetyl-D-glucosamine [1-β-4]$_n$) is a linear polysaccharide formed from repetitions of disaccharide units made up of one amino sugar and one uronic acid residue. Usually, it is referred to as hyaluronan because of the many different molecules that can be combined both physiologically and industrially.[1,2]

This biopolymer is ubiquitous in the human body and can be found in as varying environments as the skin and the extracellular matrix of cartilage. In the eye, HA is found in tear film, vitreous humor, and corneal epithelium. HA differs significantly in molecular weight throughout its wide distribution in the body, ranging from 100,000 Da in serum up to 8,000,000 Da in vitreous.[2,3]

The principal clinical role of HA in ophthalmology is its utility as a component of tear supplements, as well as its use as an artificial vitreous substitute. HA is also used in ophthalmic surgery to protect corneal endothelium during cataract surgery and to provide better graft transparency in corneal transplant surgery.[2]

Recently, growing interest in the development of new biomaterials with utility in tissue engineering and regenerative medicine has focused on HA and its derivatives. Various properties of HA, such as its molecular weight, viscosity, and hydrophobicity, can be modified in different ways to achieve new applications. A recent advance in HA manipulation is cross-linking of hyaluronan to improve its bioavailability and resistance to degradation.[4]

The intention of this review is to describe the cross-linked HA molecule to clarify its role as a tear supplement and to define potential new roles of this molecule in dry eye disease.

HA production, applications in ophthalmology, and rheology of the tear film

HA production

There are 2 competing methods presently used in the industrial production of HA. The traditional method is the extraction of HA from animal sources, such as bovine eyes and rooster combs; the second method is via the use of microbial fermentation.[1,3,5]

The traditional method for HA production is based on solvent extraction from animal tissues using cetylpyridinium chloride precipitation. After extraction, the product is passed through sterilizing filters and then precipitated once again. Finally, the HA is formulated to achieve a specific

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medical application. Beyond being a very expensive and time-consuming technique, deficiencies of this method are the low purity of the HA obtained as well as the persistence of HA-degrading enzymes.[5]

The fermentation approach gradually replaced animal extraction from the time of the early 1980s. Colonies of β-hemolytic gram-positive bacteria (group A and group C streptococci) grown in blood medium produce a slimy translucent halo consisting primarily of HA. Today, the established process applied to industrial production is based on the use of mutagenized streptococcal strains.[1,6] The D-glucuronic acid and the N-acetyl-glucosamine moieties of HA are derived from glucose-6-phosphate and fructose-6-phosphate, respectively. After many biochemical steps, hyaluronate synthase (HasA), a specific membrane-bound glycosyltransferase, links D-glucuronic acid and the N-acetyl-glucosamine to form the basic repetitive unit of HA (Fig. 1).[1,6]

HA applications in ophthalmology

HA behaves in the eye as a tissue scaffold: a supporting structure for growing cells and tissue.[7] Moreover, HA as a tissue scaffold plays an important role in eye lubrication; in fact, it demonstrates unique water-retentive and viscoelasticity properties, thanks to its random coil structure, which allows each HA molecule to hold up to 1,000 times its weight in water.[8] However, changes in temperature, pH, and shear rate can have a detrimental effect on these capabilities.[2,9]

HA has 2 distinct roles as a tear substitute: one when the eye is open and one when a blink occurs. When the eye is open, the viscosity of HA provides a protective coating that does not drain. This quality results in an improvement in the tear breakup time.[6] During a blink, HA viscosity is reduced and is therefore spread across the eye when the eyelids retreat to their original positions.

As a consequence of these characteristics, linear HA is an active ingredient in many lubricant eye drops and is used at different concentrations: that is, 0.1%, 0.15%, 0.18%, or 0.2% to stabilize the tear film and hydrate the cornea. The 0.1% concentration of HA has been the most extensively studied in artificial tears. Patients treated with 0.1% and 0.18% HA for 1 month had statistically significant relief from burning and presented reduced epithelial cell damage and consequent improved rose Bengal staining.[10–12] As a consequence of these effects, a general improvement in global symptom frequency scores has been observed.[12] Moreover, long-term use of 0.15% sodium hyaluronate-containing artificial tears was shown to reduce, efficiently and safely, ocular surface damage in dry eye patients.[13]

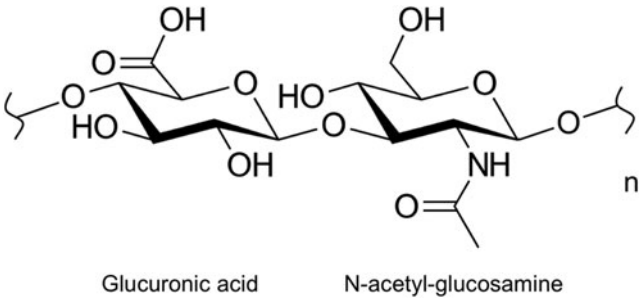

FIG. 1. The basic repetitive unit of HA. HA, hyaluronic acid.

Ophthalmic solutions of 0.1%–0.3% HA have been marketed in Japan, Europe, Australia, Russia, and USA. HA can also be used as a combination product or dual polymer (in conjunction with hydroxypropyl guar) with synergistic benefits in improving ocular surface hydration and decreasing friction.[14] These HA products appear to function more than just as ocular lubricants since they also exert pharmacological effects on the ocular surface.[6] Finally, because of its viscoelasticity, linear HA is also applied during anterior segment surgery to protect and maintain spaces during surgical manipulation.[15]

Rheology: mechanical behavior of tear and tear replacement

Fluids can be classified according to the way they behave under shear stress. A perfect fluid has no resistance to shear stress and therefore lacks viscosity. Imperfect fluids are classified as either Newtonian if their viscosity is constant for different shear rates or non-Newtonian if they change their viscosity each time a shear force is applied. If viscosity decreases when a shear force is applied, the fluid is called thixotropic fluid, and such a fluid is therefore both viscous and elastic. Physiologically, tears demonstrate thixotropy as the result of their highly complex molecular mix.[16] When tears are excreted on the ocular surface, they show a relatively high viscosity, but once moved by the eyelid margin by blinking, they lower their viscosity.[17] Most artificial tears cannot reproduce this unique characteristic.

HA is one of the most reactive non-Newtonian fluids; the viscoelasticity of HA and HA-based products varies significantly, depending on HA molecular weight and concentration.[18–20] High-molecular-weight HA is more cohesive than low-molecular-weight HA. While the former behaves as a cohesive gel, the latter behaves as a viscous polymeric fluid that is not cohesive when exposed to external high shear forces.[21] HA formulations can display both elastic and viscous behavior. HA-based tear substitutes show normal tears' thixotropic properties, with low viscosity during blinking. Weak gels and concentrated solutions that exhibit viscoelasticity, however, display a timescale-dependent deformation. On the contrary, weak gels plus concentrated solutions exhibit elastic storage of energy when the deformation timescale is short but relax into viscous flow over longer deformation timescales.[22]

Long-chained HA with a high level of intramolecular interaction has rheological characteristics more similar to normal tear film than that demonstrated by simple short-chain preparations.[23] The former molecular structure was considered essential even from the earliest days of tear replacement research. Yet, researchers soon discovered that molecular structure alone was not sufficient. As a consequence, second-generation artificial tears formulations integrated natural polymers (eg, methylcellulose derivatives) and synthetic polymers (eg, polyethylene glycol, polyvinyl alcohol, povidone, carbopol, polyguar). The disadvantage of such products consisted of the limited interchain interactions despite being long-chained and high-molecular-weight compounds. Carbomers (polyacrylic acid polymers) have been studied for years and caught on

because of their availability as linear derivatives and cross-linked molecules.[24]

From the time of their initial use, HA preparations showed great safety and efficacy as a tear substitute. HA demonstrated superior ocular comfort and improved dry eye symptoms faster than hydroxypropyl methylcellulose and carboxymethyl cellulose.[25,26] When researchers noticed its unique viscoelastic characteristics as well as its tolerability and biodegradability, both the scientific and commercial communities began to focus their efforts toward the development of HA and its derivatives. The viscoelasticity of HA leads to increased tear stability, reduction of tear removal, protective effects on the corneal epithelium, and consequently, a reduction in many dry eye symptoms.

HA modifications: cross-linked HA and its application as a tear supplement

HA modifications

HA can be used in its natural linear form, but over the past decades, the growing number of possible applications led researchers to modify the original HA structure to better suit the intended application. Specifically, HA may be subjected to derivatization processes (modification of the linear chain) or cross-linking processes [formation of covalent bonds between HA chains resulting in 3-dimensional (3D) HA networks]. HA derivatization involves sulfation and esterification processes. Sulfation of the hydroxyl groups of the HA chains determines the creation of a compound with a heparin-like activity related to the degree of sulfation.[27] On the contrary, during esterification processes, the carboxylate part of the polymer is converted into ester groups. The esterified polymer shows a reduced charge but an increased hydrophobicity.[28] As a consequence, HA increases its mechanical strength when dry, showing a reduced solubility in water depending on the degree of esterification. Esterified HA finds application as a scaffold for fibroblasts and chondrocytes growth in tissue-eingineered grafts.[28]

The most used chemical strategy to modify HAs rheological properties is cross-linking, either by direct reaction of side chains or by the addition of spacer arms to form stabilizing links between HA molecules.[6,16] Covalently cross-linked HA generates a more viscoelastic material in comparison to the original HA-based tear supplements that have low concentrations of high-molecular-weight HA. The ability of cross-linked products to maintain elastic dominance, in other words ''not to relax,'' is clearly contrasted against the ''relaxable'' solutions. A crossover into viscous-dominant behavior would eventually be found at lower frequencies [range 0.01–100 Hz (0.062–628.3 rad/s)], equating to a longer relaxation time as would be expected in a solid state.[6,22,27,29] As a consequence, the increased viscoelasticity of cross-linked HA determines a greater stability, a better resistance to degradation in stress conditions, and also a greater resistance to the enzymatic degradation by hyaluronidase.[1,16,29,30]

Cross-linking techniques are therefore very commonly applied for ophthalmologic purposes to form a HA hydrogel. These chemical modifications generally involve the primary and secondary hydroxyl groups, the carboxyl group, and the N-acetyl group.[6] The hydroxyl group may be cross-linked via an ether linkage, and the carboxyl group via an ester linkage. HA may also be treated with acid or base to obtain free amino groups, a process referred to as deacetalization. These amino groups may be cross-linked via an amide, imino-, or secondary amino bond (Fig. 2). Cross-linking reactions have been achieved under neutral, acidic, and alkaline conditions.

Auto-cross-linking and photo-cross-linking have also been described.[7] Auto-cross-linking is based on the property of HA to aggregate with itself, which is partly associated with bonding between its hydrophobic patches. These interactions are indeed weak and may be influenced by external conditions such as temperature.[31]

Other cross-linking techniques include cross-linking with polyfunctional epoxides or with glutaraldehyde and with carbodiimides.[32–35] Jeon et al. described how the mechanical properties and degradation behaviors of the cross-linked HA hydrogels may be influenced by the density and molecular weight of the cross-linker.[36] Among these cross-linking agents, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) is preferable because it can induce cross-linking of biomaterials without taking part in. EDC changes to water-soluble urea derivatives that have very low cytotoxicity.[37] EDC represents in this sense a potential biopolymer cross-linker for the fabrication of various chemically modified carriers/scaffolds for ocular tissue engineering.[38,39] Additionally, Lai et al. demonstrated that the solvent composition for carbodiimide cross-linked HA is very important in determining the water content, the mechanical strength, and the retinal pigment epithelial cell proliferative capacity.[33]

Cross-linked hydrogels based on photo-cross-linking were synthesized creating bonds between glycidyl methacrylate-HA conjugates and N-vinyl-pyrrolidinone according to a protocol modified by Leach et al.[40] It has been observed that biopolymers obtained by cross-linking HA either with adipic

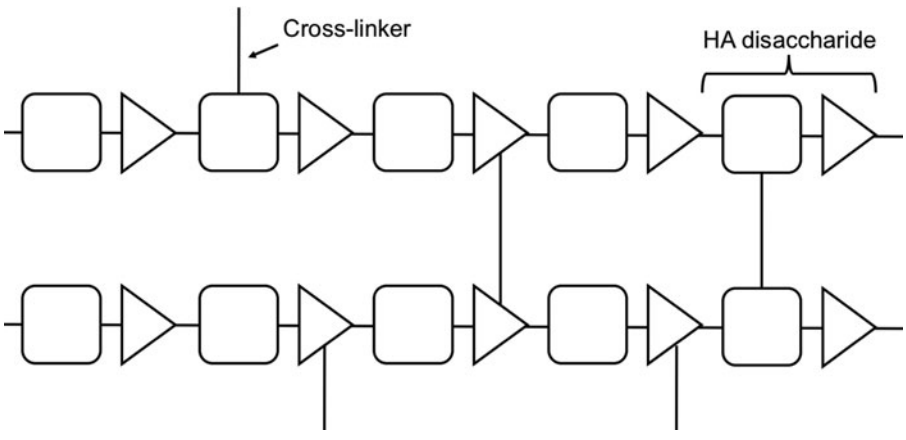

FIG. 2. Example of a cross-linked HA molecule.

dihydrazide by carboxylation with EDC after hydrazation or by photo-cross-linking with ultraviolet light and N-vinyl-pyrrolidinone are promising vitreous substitutes capable to overcome the drawbacks associated with current hydrophobic tamponade materials.[41]

Cross-linked HA as a tear supplement

The introduction of cross-linked HA as an artificial supplement, taking advantage of its physical properties, is quite recent. Particularly, as stated previously, the cross-linked HA compared with simple HA demonstrated a greater stability, that is, a better resistance on the ocular surface. Furthermore, cross-linked HA helps in the wound healing process. All these properties seem to be very useful in patients suffering from mild-to-moderate dry eye, increasing the persistence on the eye.

Yang et al. observed the efficacy of a chemically modified and cross-linked derivative of hyaluronan.[42] HA (950 kDa; Novozymes Biopolymers, Inc., Bagsvaerd, Denmark) was converted to a thiolated carboxymethyl HA (CMHA-S) by SentrX Animal Care by modifications of the literature procedures, let to form disulfide cross-links in air and then formulated into biocompatible hydrogels (CMHA-SX).

Two groups of 6 rabbits were studied: bilateral 6-mm diameter corneal epithelial abrasions were made in each of 6 rabbits of the first group and 6-mm standardized alkali burn injuries were made in the second group of rabbits. Rabbits were treated with topical administration of CMHA-SX 4 times per day in one eye, and phosphate-buffered saline was placed in the control eye of each rabbit. Closure of the corneal wound was complete by 48 h in CMHA-SX-treated eyes and by 72 h in the control eyes. The same happened for alkali burn injuries. Moreover, the epithelial thickness of the CMHA-SX gel-treated group was significantly greater than the central epithelium in the control group. In conclusion, it appeared that CMHA-SX ameliorated wound healing processes in rabbits.

Williams and Mann demonstrated how a cross-linked hydrogel based on a modified thiolated HA, xCMHA-S, ameliorated dry eye symptoms in rabbits and dogs.[43] Thiolated HA was assessed using 5,5′-dithio-bis (2-nitrobenzoic acid) (Ellman's reagent; Sigma–Aldrich). Six New Zealand white rabbits were used in this study, and 2 animals were used as negative controls. Tear breakup time was assessed with fluorescein and slit-lamp evaluation. The application of xCMHA-S gel drops increased tear breakup time compared with HA (93 ± 12 s and 71 ± 7 s, respectively), although the difference was not statistically significant ($P = 0.056$, $n = 3$). No eyes showed any sign of irritation or intolerability to any of the applied study agents. Looking at the effects of xCMHA-S gel after 2 weeks of twice daily treatment on dogs affected by keratoconjunctivitis sicca (KSC), the authors concluded that improvements in conjunctival hyperemia, ocular irritation, and ocular discharge were significantly better than those after HA treatment.

The authors concluded that cross-linked HA might lead to better patient eye health and treatment plan compliance. Moreover, they observed that this model was relevant not only for the pet dog population but also could be considered a naturally occurring spontaneous model for human dry eye.

In 2014, the same authors[30] confirmed the efficacy of xCMHA-S gel on 20 dogs affected by KSC with a masked randomized comparative study. They concluded that xCMHA-S gel showed a better therapeutic efficacy, although the Schirmer tear test improved in both groups.

Moreover, Williams et al. have published another study[44] to determine the safety and effectiveness of topical ocular administration of an xCMHA-S hydrogel in accelerating repair and closure of acute and nonhealing corneal ulcers in companion animals as a veterinary treatment and its utility as a model for therapy in human corneal ulceration. To assess safety, 2 concentrations of xCMHA-S (0.33% and 0.75%) were topically administered to the eyes of domestic pets 6 times daily for 28 days. Then, 30 dogs and 30 cats with spontaneous acute corneal ulcers were treated with either xCMHA-S (0.75%) or a non-cross-linked HA solution 3 times daily until the ulcer had healed. Moreover, 25 dogs with persistent nonhealing corneal ulcers were treated with xCMHA-S (0.75%) twice daily until the ulcer had healed. Regarding safety, xCMHA-S was very well tolerated at both concentrations with only intermittent mild conjunctival congestion and mild nonsignificant swelling of the third eyelid, and these results were confirmed histopathologically as well. Both dogs and cats showed, when treated with xCMHA-S, an accelerated closure of acute corneal stromal ulcers compared with a non-cross-linked HA; and cats showed a significant decrease in haze from pre- to post-treatment. Furthermore, in dogs, xCMHA-S improved the closure of nonhealing corneal stromal ulcers. The authors concluded that HA does appear to provide a benefit to the corneal wound repair processes.

In a recent review, Williams suggested that spontaneous KSC seen in dogs has a similar pathogenesis to human dry eye. Testing a cross-linked HA product in this model, they observed its non-Newtonian rheology as normal tear film and its tear-like properties.[45]

Fallacara et al. demonstrated the re-epithelization properties of 2 different concentrations containing a new urea-cross-linked HA: 0.02% (w/v) and 0.4% (w/v).[46] The study was conducted on both two-dimensional human corneal cells and 3D reconstructed tissues of human corneal epithelium. The 2 prototypes of eye drops developed were characterized by a good chemical–physical stability and revealed a high level of safety in ophthalmic applications. These products promoted corneal epithelial wound healing and post-wound re-epithelialization (increased level of cyclin D1 have been described) similar to negative controls (not damaged tissues) and superior to damaged untreated tissue (positive controls). The encouraging results of the investigation support further research into the biological activity of artificial tears containing urea-cross-linked HA.

The first study concerning the use of cross-linked HA in humans was published in September 2017. Cagini et al. compared 20 patients with Sjögren's syndrome-related dry eye (SSDE) with 20 controls, before and 5, 30, and 60 min after instillation of eye drops.[47] Cross-linked HA was synthesized using EDC. The surface regularity index (SRI) and surface asymmetry index (SAI) varied significantly in the SSDE groups after 60 min for the SAI, and after 30 and 60 min for the SRI from instillation of cross-linked HA. Instead, in healthy subjects, the instillation of the 2 different tears substitute, HA or cross-linked HA, did not show statistically significant differences between the SRI and SAI values, except at 5 min for the SAI. No patients reported stinging or any other adverse ocular event. These results showed that in patients with major tear film instability and greater ocular surface irregularities, the stability of the tear

film was much higher after the use of cross-linked HA than that with HA.

Recently, Postorino et al. published their results about the use of cross-linked HA and coenzyme Q10 in treating patients with mild-to-moderate dry eye.[48] This was a randomized, single-masked, parallel group, and comparative study. Forty patients with mild-to-moderate dry eye disease were enrolled. Twenty patients received cross-linked HA + coenzyme Q10 (group A), and the remaining 20 patients received 0.15% HA (group B). After treatment, the ocular surface disease index score significantly decreased in groups A and B ($P < 0.01$ and $P < 0.05$, respectively); but group A showed a significantly greater decrease. Corneal staining decreased in both groups, with lower scores in group A. Meibomian gland disease improved significantly in group A. Epithelial cell reflectivity, keratocytes, and stromal matrix parameters improved significantly only in group A.

The authors concluded that cross-linked HA could increase the stability, adhesiveness, and permanency of coenzyme Q10 on the ocular surface, and the patients suffering from dry eye may benefit from the greater permanence of the cross-linked HA and of the antioxidant activity of coenzyme Q10. Conclusions of this work are limited because natural HA was compared with a mixture of Q10 and cross-linked HA. The lack of proper controls does not allow inferences about the benefits of cross-linked HA versus natural HA. Moreover, the tear film stability was not measured.

Future Perspectives

Dry eye disease is a multifactorial disease, and its prevalence varies between 5% and 33% depending on the different diagnostic criteria and the population studied.[49–53] Dry eye is characterized by tear film instability, visual disturbance, and potential damage to the ocular surface.[49] Usually, dry eye disease is accompanied by increased tear osmolarity that stimulates the production of inflammatory mediators on the ocular surface.[52,53] HA, a natural glycosaminoglycan, is a component of the tear film[13] and hydrates and lubricates the ocular surface.[54] Moreover, HA possesses intrinsic water retention properties and viscoelasticity and helps in the healing of corneal and conjunctival epithelium.[55,56] Safety and efficacy of HA for the treatment of signs and symptoms of moderate-to-severe dry eye syndrome have been demonstrated in human studies.[56] It has also been shown that osmoprotective eye drops containing HA determine a reduction of inflammation of the ocular surface with consequent improvement of the quality of corneal and conjunctival epithelium.[57–59]

Cross-linked HA has been developed to improve the permanence of this molecule on the ocular surface, reducing the number of instillations and increasing patients' compliance.

There are few studies regarding cross-linked HA, especially in humans,[47,48] but these preliminary reports have confirmed that it could represent a new valid option for treatment of patients affected by mild-to-moderate dry eye.

Glaucoma patients frequently suffer from dry eye disease, both as a consequence of preservatives and as a direct action of the therapeutic compound.[60–62] It has been largely demonstrated that the number of glaucoma patients suffering from dry eye increases with the number of medications used and with disease duration.[63] In this particular population, the use of preservative-free medications and tear supplement[64–67] is very useful to reduce dry eye symptoms. Particularly, cross-linked HA, for its stability and persistence over the ocular surface, may represent a valid alternative.

Summary

There is still an unmet medical need for next-generation HA products that can outperform the available artificial tear options in terms of increasing ocular persistence, decreasing dosing frequency, improving tear film, and alleviating symptoms associated with acute and chronic dry eye disease.

Cross-linked HA is a more viscoelastic material, with a non-Newtonian behavior. It is well tolerated *in vitro* and *in vivo* and exhibits longer resistance on the ocular surface and a reduction of dry eye symptoms on patients affected by dry eye disease. Preliminary results about these new artificial tear supplements are very encouraging, but further studies are needed to better clarify the possible application of this molecule, not only in patients suffering from dry eye disease but especially in medically treated glaucoma patients who frequently experience severe dry eye symptoms due to chronic exposure to hypotensive medications.

Availability of Data and Materials

All the data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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