Original Article

Use of 2% hydroxypropyl methylcellulose to prevent the corneal swelling during the *in vitro* mechanical characterization

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Abstract

The purpose of this study was to establish adequate conditions for the storage and handling of the corneal tissue destined to be *in vitro* tested for the characterization of the mechanical properties. Twenty-eight rabbit eyes were divided into 4 groups. Group A (7 eyes) 24 hours preserved in refrigerated (4ºC) NaCl 0.9% solution before testing. Group B (7 eyes) immediately tested. In both groups, to prevent both swelling and dehydration, a preparation of hydroxypropyl methylcellulose 2% (HPMC 2%) was applied. Group C (7 eyes) 24 hours preserved in refrigerated (4ºC) NaCl 0.9% solution before testing. Group D (7 eyes) immediately tested. In both groups, preparation of HPMC 2% was not applied. Regarding the mechanical response, groups A and B (handled with HPMC 2%) showed similar Cauchy stress-stretch curves and there were no statistically significant differences at 5%, 10% and 15% strain between them, which means that both showed similar mechanical behavior. The same result was obtained between groups C and D (without HPMC 2%). However, for coupled groups AB (with HPMC 2%) and CD (without HPMC 2%) statistically significant differences at 10% and 15% strain were observed. On the other hand, when grouped by storage time, statistically significant differences were found between groups A and C (24 hours stored with and without HPMC 2%, respectively), as well as between groups B and D (immediately tested with and without HPMC 2%, respectively) at 15% strain. Nevertheless, if coupled groups were considered, between groups AC (24 hours in NaCl 0.9% before tested) and BD (immediately tested) no statistically significant differences were obtained. In addition, the Cauchy stress-stretch curves of groups without HPMC 2% showed a decreasing slope of the linear part (strain > 8%) of the graph during the experiment. In the author´s opinion, this fact could be due to some level of swelling happened in those samples during handling or mechanical testing. In summary, the use of a preparation of HPMC 2% during the handling of the tissue from excision to testing seems to prevent both swelling and dehydration. Moreover, cold storage of the eye in NaCl 0.9% solution for 24 hours before testing does not modify the mechanical response.

Keywords

Rabbit, cornea, conservation, hydroxypropyl methylcellulose, biomechanical properties

Introduction

The cornea is the most powerful refractive component of the eye. Its function as an optical lens is based on its morphology and transparency, both due to the corneal microstructure. Among the different corneal layers, the stroma constitutes about 90% of the whole corneal thickness and is the main responsible of the mechanical properties of the cornea. However, the active regulation of corneal hydration is achieved by epithelium and endothelium. Therefore, regardless of the cause, any change in one of these two barriers induces a modification of the stromal hydration state and thereby of its microstructure, including sample preparation for the *in vitro* tests performed to asess the mechanical properties of the corneal tissue. Consequently, all these facts can result in a significant alteration of the mechanical properties of the corneal tissue.[1,2]

Nowadays, most tests for the mechanical characterization of the corneal tissue are performed *in vitro* and it is necessary to remove the tissue sample from the eye.[3-5] Furthermore, such tests are not often immediately performed and the samples are stored by different methods during transport from the collection site to the laboratory for the subsequent experimental characterization. Whatever the preservation method chosen, the aims are maintaining the integrity of the corneal tissue, extending the storage time, and avoiding contamination. During the mechanical testing, dehydration is the most important factor and it must be prevented, thus the tissue is usually tested within a moist chamber. Therefore, validation of new methods of conservation and handling is needed to ensure good preservation of the tissue, without modification of the corneal water content from collection to its final use in the lab.

The purpose of the present study is to assess the use of hydroxypropyl methylcellulose 2% during the execution of *in vitro* uniaxial tensile tests in moist atmosphere, with corneal strips immediately obtained or after 24 hour-storage of eyes in NaCl 0.9% solution at 4ºC. This new procedure is proposed to prevent corneal swelling due to storage or handling conditions, thus preventing the modification of its mechanical properties.

Material and methods

Study design and samples preparation

Twenty-eight freshly enucleated New Zealand rabbit eyes (animal weight 2-2.5 kg) were retrieved from the local abattoir an hour post-mortem. All eyes presented a clear cornea with intact epithelium. For a good maintenance, during the whole process the eyes were preserved at 4°C in a moist chamber.

For the mechanical testing, rabbit eyes were divided into four groups. Group A, seven eyes 24 hours preserved in refrigerated (4ºC) NaCl 0.9% solution before testing. Group B, seven eyes immediately tested. In A and B groups, to keep the hydration of the corneal tissue and to prevent the swelling caused by the moist environment, a preparation of hydroxypropyl methylcellulose 2% (HPMC 2%, Methocel® 2%, OmniVision AG, Puchheim, Germany) was applied on 4 the sides of the corneal strip. Group C, seven eyes 24 hours preserved in refrigerated (4ºC) NaCl 0.9% solution before testing. Group D, seven eyes immediately tested. In C and D groups, preparation of HPMC 2% was not applied.

For the sample preparation, a circular area including cornea and a ring of sclera were cut off from the enucleated eyes. Afterwards, a corneal strip with dog-bone shape (including central cornea and sclera) and a width/length ratio around 1/7 was removed with a double-bladed scalpel in the superior-inferior direction, to preserve the uniaxial tension hypothesis. The corneal thickness of each strip was obtained as the average of 5 measures recorded with a solid contact ultrasonic probe (DGH 500 Pachette™; DGH Technologies Inc., Exton, USA). Furthermore, in A and C groups the corneal thickness was determined previously and after the 24 hours preservation of the eye in refrigerated (4ºC) NaCl 0.9% solution. The strip width was accurately measured by placing the corneal strip in a micrometer (Mitutoyo Absolute Digimatic Series 227, Mitutoyo Inc., Illinois, USA). Three measurements were taken for each sample in order to assess the sample width homogeneity.

Mechanical characterization

The corneal strips were clamped vertically between the jaws and uniaxial tensile tests were performed under displacement control on an Instron 5548 Microtester (Illinois Tool Works Inc., Glenview, USA) with a 10 N full scale load cell. In order to avoid specimen drying, an ultrasonic humidifier was used, which provided a subcooling steam and kept a constant temperature of 25°C (Figure 1). A testing velocity estimated as $0.2L$x60/100 mm·min^{-1}, where L is the initial sample length and 0.2 is the deformation rate, was maintained throughout the test and for all specimens. Load and displacement were recorded until complete sample rupture. The strip elongation can be expressed in both stretch (») and percentage elongation: (»-1)*100%. The stretch data was computed as »=$(L_0+\Delta L)L_0^{-1}$, where L_0 is the initial length between clamps and ΔL is the upper clamp displacement. The Cauchy stress was obtained as Ã=N»/CSA, where N is the applied load and CSA the initial cross-sectional area of the specimen.

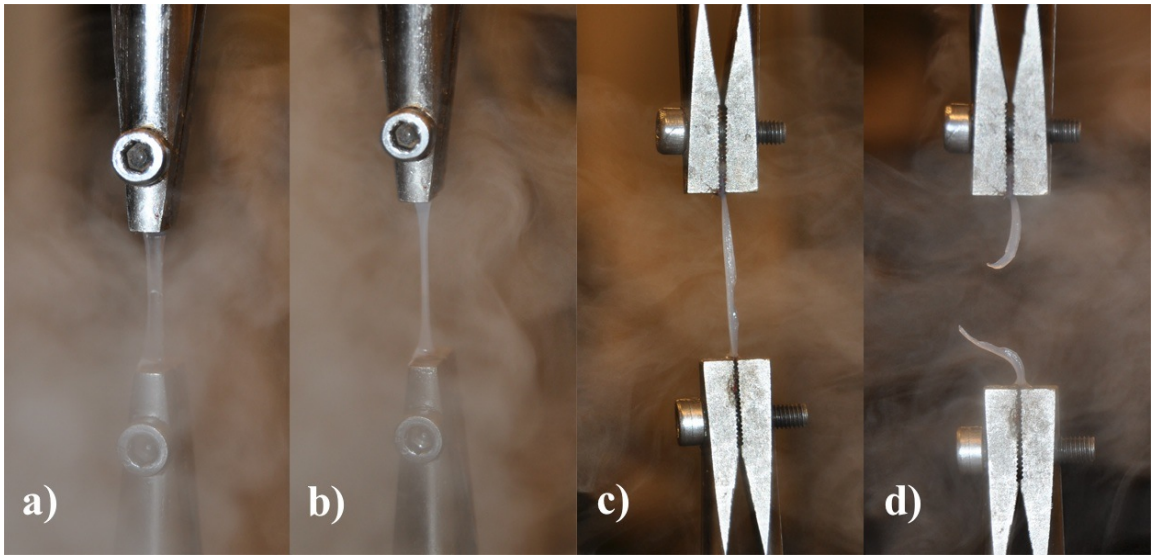

Figure 1. Strip of corneal tissue after being clamped by the jaws (a), tensile test evolution (b,c) and tissue rupture (d).

Statistical analysis

After the stress-stretch curves of individual samples were acquired, they were averaged to obtain the mean response of each group. Subsequently, a statistical analysis was performed using MatLab 2010a (The MatWorks Inc., Natick, USA) and SPSS 19.0 for Macintosh (SPSS Inc., Chicago, USA). Normal distribution for continued variables in groups was evaluated by the Kolmogorov-Smirnov test; *p* values less than 0.05 were considered significant (no normal distribution). Stress values at 5%, 10% and 15% strain (± standard deviation) were calculated for all samples. Student's t-test (if normal distribution) or Wilcoxon test (if no normal distribution) were used to compare the strip thickness values and Cauchy stress (Ã) at stretch »=1.05 (5% strain), »=1.10 (10% strain) and »=1.15 (15% strain) between groups A,B (with HPMC 2%); C,D (without HPMC 2%); A,C (24h NaCl 0.9%); B,D (immediately tested); AB,CD (with HPMC 2% *vs* without HPMC 2%) and AC,BD (24h NaCl 0.9% *vs* immediately tested). Furthermore, the corneal thickness was also compared before and after preserving the eye 24 hours in NaCl 0.9% solution (AC).

Results

The average strip thickness, width and length between the jaws values (± SD) of groups A (24 hours in NaCl 0.9% before tested with HPMC 2%), B (immediately tested with HPMC 2%), C (24 hours in NaCl 0.9% before tested without HPMC 2%), D (immediately tested without HPMC 2%), AB (with HPMC 2%), CD (without HPMC 2%), AC (24h in NaCl 0.9%) and BD (immediately tested) are shown in Table I. For the thickness, no statistically significant differences were found between individual groups (A,B p=0.328; C,D p=0.188; A,C p=0.122; B,D p=0.788), using HPMC 2% or not (AB,CD p=0.327), nor between groups 24 hours stored or immediately tested (AC,BD p=0.529). Furthermore, there were no statistically significant differences between the thickness observed previously (0.389 ± 0.017 mm) and

after the 24 hours preservation in refrigerated (4ºC) NaCl 0.9% solution (0.395 ± 0.015 mm) (AC p=0.193).

Table I. Average strip thickness, width and length values (mm ± SD).

	Thickness	Width	Length
Group A	0.387 ± 0.016	2.030 ± 0.070	13.828 ± 0.984
Group B	0.390 ± 0.019	2.131 ± 0.204	12.993 ± 0.790
Group C	0.401 ± 0.011	1.919 ± 0.096	13.557 ± 0.481
Group D	0.387 ± 0.019	1.946 ± 0.232	13.574 ± 0.338
Group AB	0.389 ± 0.017	2.085 ± 0.151	13.378 ± 0.807
Group CD	0.395 ± 0.016	1.932 ± 0.164	13.566 ± 0.406
Group AC	0.395 ± 0.015	1.970 ± 0.097	13.682 ± 0.667
Group BD	0.389 ± 0.018	2.039 ± 0.224	13.284 ± 0.550

The Cauchy stress-stretch curves (Ã-») obtained are depicted in Figure 2 for each group of study and they represent the mechanical behavior of these. In each curve can be distinguished three major regions: 1) the toe region, represents "un-crimping" of the collagen fibrils, 2) the linear region, the collagen fibrils become uncrimped and they begin to stretch making the tissue stiffer, and 3) the yield and failure region, the accumulation of damage due to individual fibril failure causes loss of stiffness and then the tissue begins to fail. In our study, all the curves were truncated before complete failure of the tissue at a strain of 15% and the mean curve was obtained afterwards.

To perform the statistical analysis, the stress data for strains of 5%, 10% and 15% were considered. These values (± SD) are listed in Table II. All groups showed a normal distribution (p>0.05) and the p values obtained are represented in Table III. For 5%, 10% and 15% strain, there were no statistically significant differences between groups A and B (with HPMC 2%), which means that both showed similar mechanical behavior. A similar result was obtained between groups C and D (without HPMC 2%) but between coupled groups AB (with HPMC 2%) and CD (without HPMC 2%) statistically significant

differences at 10% and 15% strain were observed. The stress at 5% and 10% strain did not show statistically significant differences between groups A and C (24 hours in NaCl 0.9% before tested with and without HPMC 2%, respectively), while for values of stress at 15% strain, the difference among these two groups was significant. A similar result was obtained between the group B and D (immediately tested with and without HPMC 2%, respectively). If coupled groups were considered, between groups AC (24 hours in NaCl 0.9% before tested) and BD (immediately tested) no statistically significant differences were obtained.

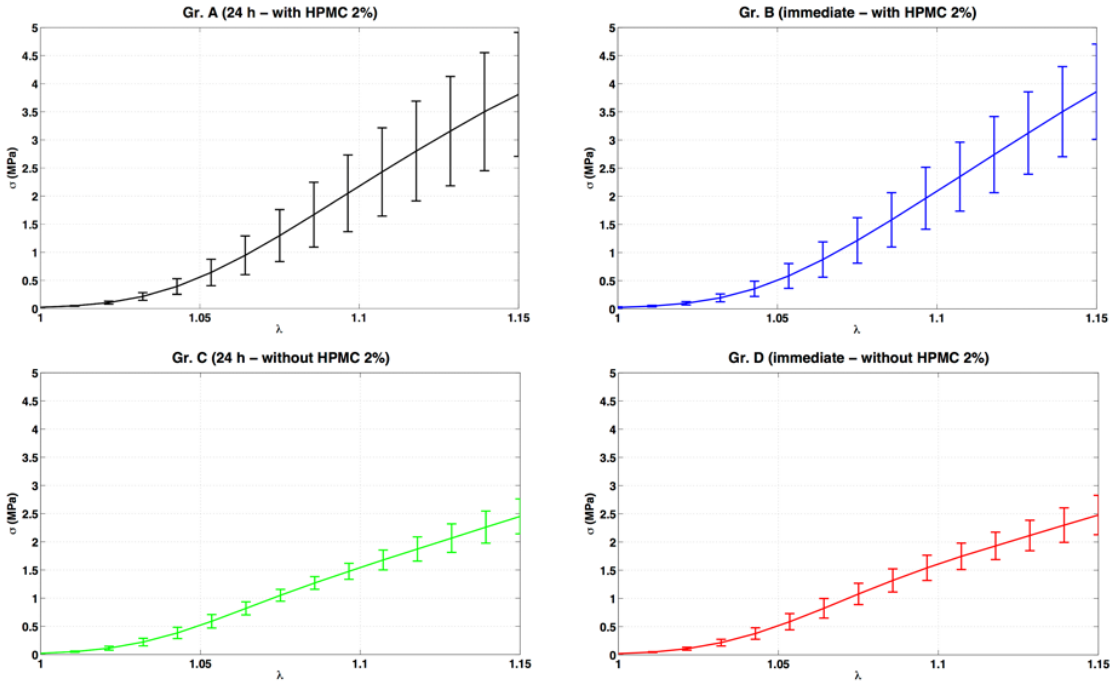

Figure 2. Average experimental Cauchy stress-stretch curves of the corneal strips groups.

Table II. Average stress values (MPa ± SD) for 5%, 10% and 15% strain.

Strain (Stretch)	Group A	Group B	Group C	Group D
5% (»=1.05)	0.55 ± 0.15	0.50 ± 0.16	0.52 ± 0.08	0.51 ± 0.12
10% (»=1.10)	2.18 ± 0.51	2.09 ± 0.49	1.55 ± 0.11	1.62 ± 0.19
15% (»=1.15)	3.81 ± 0.85	3.87 ± 0.73	2.45 ± 0.24	2.48 ± 0.31

Table III. *p* values between the groups at 5%, 10% and 15% strain.
Statistically significant values are underlined.

Strain (Stretch)	5% (»=1.05)	10% (»=1.10)	15% (»=1.15)
p (A,B)	0.778	0.932	0.810
p (C,D)	0.942	0.453	0.903
p (A,C)	0.786	0.086	<u>0.026</u>
p (B,D)	0.931	0.091	<u>0.006</u>
p (AB,CD)	0.974	<u>0.005</u>	<u>0.000</u>
p (AC,BD)	0.823	0.797	0.551

Regarding the mechanical response, groups A and B (with HPMC 2%) showed a similar behavior, and this was stiffer than that shown by groups C and D (without HPMC 2%), that also presented a similar mechanical behavior between them. In groups 24 hours preserved in refrigerated NaCl 0.9% solution before testing (A and C), the group A (with HPMC 2%) showed stiffer behavior than the group C (without HPMC 2%). In groups immediately tested (B and D), the group B (with HPMC 2%) showed stiffer behavior than the group D (without HPMC 2%) (Figures 3). When groups were coupled by use of HPMC 2%, AB (with HPMC 2%) showed stiffer behavior than CD (without HPMC 2%). However, when grouped by storage time, AC (24h in NaCl 0.9%) showed similar behavior than CD (immediately tested) (Figures 4 and 5).

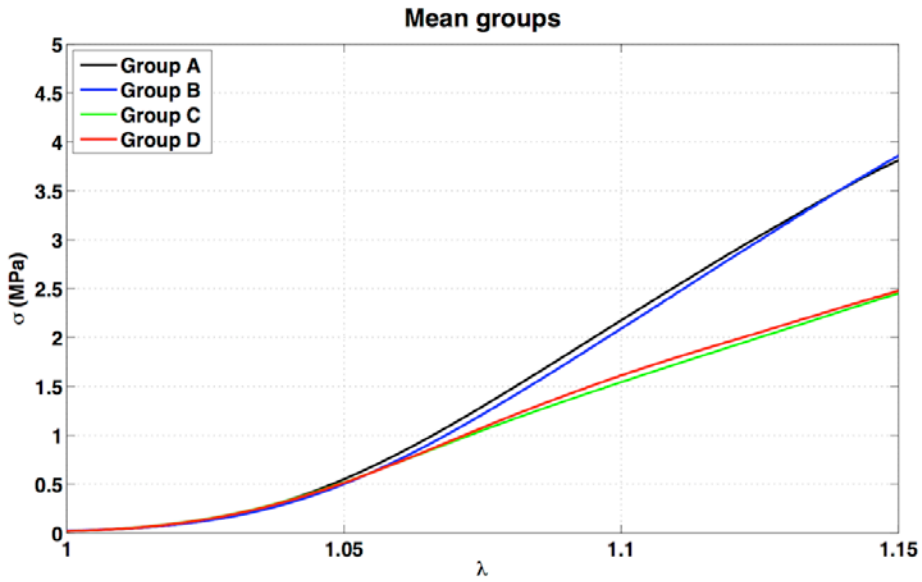

Figure 3. Experimental Cauchy stress-stretch curves (mean value of the groups). Group A (24h in NaCl 0,9% and tested with HPMC 2%), group B (immediately tested with HMPC 2%), group C (24h in NaCl 0,9% and tested without HPMC 2%) and group D (immediately tested without HMPC 2%).

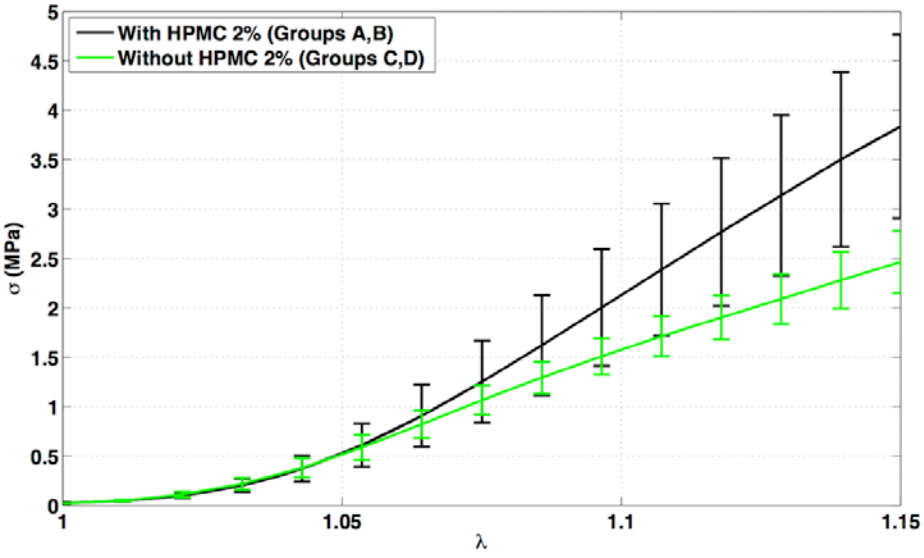

Figure 4. Average experimental Cauchy stress-stretch curves (groups with and without HPMC 2%).

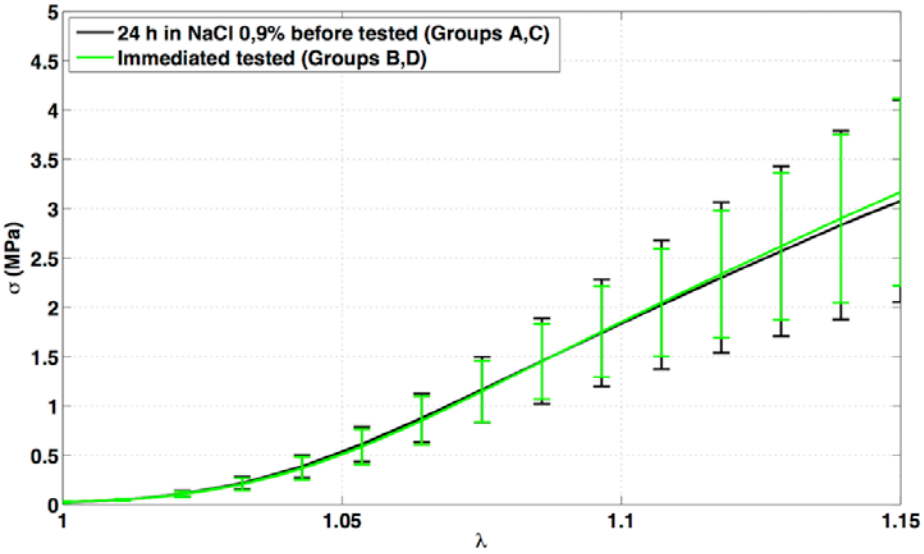

Figure 5. Average experimental Cauchy stress-stretch curves (groups 24 h in NaCl 0.9% before tested and immediately tested).

Discussion

In the present study, we assess the use of HPMC 2% during *in vitro* uniaxial tensile tests, with corneal strips immediately obtained or after 24 hour-storage of eyes in NaCl 0.9% solution at 4ºC. The purpose is to establish a standardized procedure to prevent corneal swelling, which is known to modify the mechanical properties of the tissue. Furthermore, this protocol would guarantee the quality of results obtained by mechanical tests with corneal tissue after a span of time from the eye collection.

In general, the collection of corneal tissue for *in vitro* mechanical tests is carried out by two methods: enucleation or *in situ* corneal excision. Enucleation previous to corneal excision is usually performed because it warrants better conservation in those cases that the cornea has to be stored for some time up to its final use.[6] Shortening this time is imperative to avoid altering both structure and biomechanical properties.[2,7] In our study, the eyes were enucleated in the local abattoir just after the animal death, being transported in moist chamber at 4ºC up to the moment of the mechanical test, or stored in NaCl 0.9%

solution for 24 hours, both within the first hour after collection. Cold storage in moist chamber at 4ºC is one of the most used methods for corneal tissue storage. In spite of preserving the tissue for a shorter time than other methods such as cryopreservation or organ culture, storage in moist chamber allows a good preservation of the structure and composition of the corneal tissue.[6,8] This method is frequently used in the literature for preserving the corneal tissue before performing mechanical tests.[2,3]

Handling of the corneal tissue before and during mechanical characterization is as important as its preservation. As happens *in vivo*, corneal hydration is regulated by the epithelium and endothelium, while stroma is the main responsible of the biomechanical properties of the whole cornea. Therefore, preserving the integrity of these three layers during storage and handling is mandatory to maintain the properties of the corneal structure.[1,2,5,9] However, during the *in vitro* uniaxial tensile test, when the cornea is excised from the eye and the strip is cut from the cornea, a rapid process of dehydration occurs when the tissue is not in a moist environment, or the opposite process, swelling, happens in the presence of saline solution, liquid or vapor. With the purpose of preventing this, some solutions as dextran, riboflavin/dextran, Optisol-GS have been used for *in vitro* mechanical tests.[2,4] Nevertheless, it has been proved that dextran and Optisol-GS produce dehydration and hydration of the corneal tissue, respectively, modifying its biomechanical properties.[2,10] On the other hand, other isotonic (0,9% NaCl solution, phosphate buffer saline, ophthalmic balanced salt solution), hypertonic (12% NaCl), hypotonic (distilled water) and neutral solutions (mineral oil) also have been previously used as bathing solution during *in vitro* strip extensiometry tests of the scleral and corneal tissue, in order to prevent swelling (or dehydration) of the samples and keeping their thickness (hydration state) constant.[1,5,11,12] However, any solution has been proposed as a perfect bathing solution without effect over the physic-chemical properties of the corneal tissue and more investigations are required to assess other preventing solutions to perform *in vitro* mechanical tests.

Other components as hyaluronic acid and methylcellulose, whose effects over the corneal tissue have been more studied and are used routinely in ophthalmic surgery, could be more adequate. Regarding the hydroxypropyl methylcellulose, it is unclear its convenience for the treatment of the dry eye in 0.5% solution on the epithelium, since it may induce physic-chemical and functional alterations of the mucous and precorneal tear film components.[13] Nevertheless, it seems to supply a proper coating and shield on the corneal surface, reducing the evaporation of the tear film and maintaining the corneal turgescence.[14] This compound has also been proven at 4% to achieve a firm adhesion of the eyelids, avoiding ocular surface drying and protecting it mechanically. Moreover, no adverse effect during non-ocular surgeries has been observed.[15] With respect to the endothelium, no significant change in intraocular pressure was observed capable of modifying corneal hydration during cataract extracapsular surgery[16] and its efficiency to prevent swelling and endothelial cell damage induced by the surgery has been confirmed.[17,18] In the present study, we propose the use of HPMC 2% as an alternative to ensure a good preservation of the tissue without modifying the water content of the cornea, during performing any mechanical test that may imply an alteration of the corneal structure. In a previous study, we used the HPMC 2% with this purpose during *in vitro* uniaxial tensile tests performed to assess the effect of the corneal collagen crosslinking in porcine eyes.[3] To the author's knowledge, there are no reported studies comparing the use of HPMC 2% during the execution of *in vitro* uniaxial tensile tests with corneal strips immediately obtained or after 24 hour-storage of eyes in NaCl 0.9% solution at 4ºC.

The thickness of all samples was analyzed previously to mechanical testing and no statistically significant differences were found between groups using HPMC 2% or not, nor between groups 24 hours stored or immediately tested. However, while the Cauchy stress-stretch curve remains with a nearly constant slope (until 1.15 stretch) in both groups where HPMC 2% was applied, the change in slope shown by groups without HPMC 2% during the experiment was not expected. This fact could be due to a failure in the microstructure at an earlier stage (1.06 stretch) with a progressive loss of stiffness as the test

progresses. Furthermore, we found statistical significant differences between groups with and without HPMC 2% but not between groups immediately tested and 24 hours kept in NaCl 0.9%. In the author´s opinion, some level of swelling could have happened in those samples without HPMC 2% during handling or due to the water vapor emitted by the ultrasonic humidifier in the mechanical testing. This device is needed to maintain an homogeneous humidity and temperature, and the use of HPMC 2% during testing seems to provide a shield on the tissue surfaces, which limits the vapor absorption and remains constant the hydration level of the sample.

In summary, the use of HPMC 2% during the handling of the corneal tissue from excision to *in vitro* mechanical testing, is a new alternative to prevent both swelling and dehydration even for experiments in a moist environment. Moreover, it has been demonstrated that the corneal mechanical behavior is not modified after storage of the eye in NaCl 0.9% solution for up to 24 hours before its mechanical characterization.

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Conflict of interest

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