# Hyaluronic acid combined with mannitol to improve protection against free-radical endothelial damage: Experimental Model

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***Purpose:*** To evaluate the protective properties of combined sodium hyaluronate 2% and mannitol 0.5% (Visiol) on the corneal endothelium in the presence of oxidative stress induced by hydrogen peroxide ($H_2O_2$).

***Setting:*** Instituto Oftalmológico de Alicante, Universidad Miguel Hernández, Alicante, Spain.

***Methods:*** This was an exploratory randomized controlled parallel-group, masked-assessor study of 3 sodium hyaluronate-based ophthalmic viscosurgical devices (OVDs): Visiol, Healon (sodium hyaluronate 1%), and Viscoat (sodium hyaluronate 3%–chondroitin sodium 4%). The OVDs were tested for protective effects on the endothelium following oxidative stress induced by $H_2O_2$ at increased concentrations: control (lactated Ringer's solution), 1 mM, 10 mM, and 100 mM. Groups without OVD were used as controls at the same concentrations of peroxide. Each animal received the same treatment in both eyes (10 eyes per group). Endothelial cell lesion was assessed using the Janus green photometry absorbance technique.

***Results:*** At 10 mM peroxide concentration, the value of endothelial cell lesion was significantly lower in the Visiol (16.8%, $P = .0056$), Healon (22.2%, $P = .0302$), and Viscoat (21.6%, $P = .0336$) groups than in the control group (29.4%, no OVD). There was a trend in favor of Visiol to more efficiently reduce cell lesions of the endothelium, than Healon ($P = .055$) and Viscoat ($P = .1013$). Values of endothelial cell lesion at peroxide concentrations of 1 mM and 100 mM showed the same trends than those observed at 10 mM.

***Conclusions:*** All of the OVDs tested efficiently reduced endothelial lesions against free radicals compared with the control group in which no OVD was used. The following sequence for the efficacy of endothelial cell protection was established: Visiol > Viscoat > Healon > no OVD.

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Cataract surgery is currently the most frequently performed surgical procedure. Some of the most important conditions for a successful cataract operation, as judged by the surgeons, are to maintain a deep anterior chamber, especially to facilitate the insertion of intraocular lens, and to protect the intraocular tissues from the surgical maneuvers. These conditions are essential for microsurgical manipulation in the spatially limited anterior eye segment and ultimately serve to protect the corneal endothelial cells.[1]

During phacoemulsification, the tip of the probe oscillates at an ultrasonic frequency. The ultrasonic wavelengths pass through the aqueous humor, generating tiny cavitation bubbles that expand and implode liberating energy and heat.[2] The energy and heat released lead to the formation of extremely reactive free radicals that may harm the cells of the corneal endothelium.[3–7] Hydrogen peroxide ($H_2O_2$) is normally found at extremely low levels in the aqueous humor (0.025 mM). However, it has been shown that



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peroxide levels higher than 0.3 to 0.5 mM are quickly toxic to the endothelium and promote corneal edema.[4]

Most of the commercially available ophthalmic viscosurgical devices (OVDs) are sodium hyaluronate (SH)-based products, which have already been shown to have a free radical scavenging effect. However, the scavenging reaction on SH is followed by a breakdown of the molecule, thus reducing the protective effect of SH to the corneal endothelium.

As a result, a new generation of OVDs was developed that contains mannitol, a scavenger of free radicals that are produced during phacoemulsification of the nucleus. This characteristic is believed to enhance protection to the endothelium and maintain the physical–chemical characteristics of SH throughout surgery. This is particularly important in patients with a hard nucleus, in which duration of phacoemulsification and thus production of free radicals increase.

The objective of this study was to evaluate the protective properties of combined sodium hyaluronate and mannitol versus existing products on the endothelium in the presence of oxidative stress induced by $H_2O_2$. This study was performed according to an experimental model previously described in the literature.[4]

## Materials and Methods

### *Investigational Plan*

This was an exploratory randomized controlled parallel-group masked-assessor study. Three OVDs (Visiol, Healon, and Viscoat) were tested for protective effects on the endothelium following oxidative stress induced by $H_2O_2$ at different concentrations: control (lactated Ringer's solution) and 1 mM, 10 mM, and 100 mM (Table 1). Groups without OVD (no OVD) were used as controls at the same concentrations of peroxide. Each animal received the same treatment in both eyes (10 eyes per group).

Each group of 5 animals was randomly allocated to the different treatment groups (16 groups of 5 animals). The randomization list was created by the statistician using the valid computer-program Rancode (IDV).

For the randomization of rabbits, an independent person randomly chose 5 rabbits (1 group) to the surgeon who assigned the treatment group in accordance with the randomization list provided by the statistician. The surgeon was not blind to the treatment given because the products have different presentations and physical characteristics (eg, volume, viscosity). A different person (assessor) who was masked to the treatment carried out the assessments and measurements. The same surgeon carried out all these procedures in all the animals. The same surgical technique was used in all the animals. The same assessor performed the same assessment in all the rabbits or was always involved in the same steps for a particular assessment.

### *Test Products*

Visiol contains SH 2% (molecular weight 1.8 $10^6$ Dalton [Da]), mannitol 0.5% (wt/vol), sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, and water for injection. It is an isotonic solution adjusted to pH 7.3 and does not contain any preservative. Healon (SH 1%, molecular weight $4 \times 10^6$ Da) and Viscoat (SH 3%, molecular weight $0.5 \times 10^6$ Da and chondroitin sulfate 4%, molecular weight 22.5 Da) were used as controls.

### *Animals*

Care of the animals conformed to the Association for Research and Vision in Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research, and the study was approved by the local ethics committee. The required number of animals was selected from those supplied following veterinary examination for general health and

**Table 1.** Study overview.

| Group | OVD | | | |
|---|---|---|---|---|
| | None | Visiol | Healon | Viscoat |
| Control (lactated Ringer's) | X | X | X | X |
| Hydrogen peroxide | | | | |
| 1 mM | X | X | X | X |
| 10 mM | X | X | X | X |
| 100 mM | X | X | X | X |

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ocular defects. Selected animals were healthy and free of ocular defects. The animals were ordered weighting approximately 2 kg and 7 to 9 weeks of age with female animals nulliparous and nonpregnant. Animals were individually housed in grid-bottomed metal cages suspended over trays. Each cage tray held absorbent material, which was inspected daily and changed as necessary.

Animal room temperature and relative humidity was set at 15°C to 21°C and 45% to 65%, respectively. Fluorescent lighting provided a 24-hours cycle of 12 hours light/12 hours dark. An antibiotic-free pelleted diet was obtained from Harlan Iberica, SA. Diet and tap water via bottles were available ad libitum.

### *Experimental Procedure*

The animals were anesthetized with intramuscular ketamine chlorhydrate (50 mg/kg) and topical tetracaine 1%. Then, the procedure was carried out as described in Table 2.

### *Janus Green Photometry Absorbance Technique*

This method allows a quantitative measurement of both endothelial denuded areas and those areas in which endothelial cells are affected by a nonreversible process of oxidative degeneration. Janus dye selectively stains such areas and not viable corneal endothelial cells. The endothelial side of the corneal button was incubated with 200 µL of a 1% Janus green dye solution (Sigma) for 90 seconds. Then, the vital stain was washed out from the endothelium for 2 minutes with balanced salt solution and trephination of a corneal button was done. The Janus green dye that was fixed to the denuded or degenerated areas of the corneal endothelium was removed from the corneal tissue by incubating the corneal button for 90 seconds in 1 mL of absolute alcohol. The alcohol sample from which the Janus green dye was removed was then diluted at 10% to perform a photometry measurement using a Smart-Spect 3000 spectrophotometer (Bio-Rad).

A standard curve of absorbance was previously established using central corneal buttons 4.0, 6.5, 8.0, and 9.0 mm diameter (n = 10 at each diameter that were trephined after complete alteration of the whole endothelium with absolute alcohol). After they were rinsed, the corneal endothelial cells were stained with Janus green B 1% for 90 seconds. The dye taken by each corneal was eluted in 1 mL of absolute ethanol in an Eppendorf disposable tube. Complete extraction of the stain was achieved after 90 seconds. Photometric measurements of the elute were performed with the spectrophotometer at 650 nm; absolute ethyl alcohol was considered the blank value. The 9.0 mm diameter value was taken as 100% damage, and a 9.0 mm diameter normal cornea (not altered with alcohol) was taken as 0% damage (n = 10). With all these data, a standard graph was established. Subsequently, the 9.0 mm trephine was used for all the experiments. The photometry values in the problem corneas were obtained by extrapolating from the standard values using a computer program (Excel 2002 software, Microsoft), allowing the amount of endothelial damage to be expressed as a percentage.

The maximum peak value of the spectrophotometric measurement of Janus green absorbance was assessed (650 nm), and all further measurements were taken at this wavelength.

**Table 2.** Experimental procedure.

| Step | Description of Procedure |
|---|---|
| 1 | 100 µL of aqueous humor was removed from the eye of the animal with a precision aspiration pump (Hamilton) and a syringe with a 30-gauge needle inserted through the corneoscleral limbus. |
| 2 | A 1 mm stab limbar paracentesis was done, and OVD was introduced into the AC until complete filling. Ease of injection was assessed. |
| 3 | Wait for 1 minute. |
| 4 | An I/A cannula was introduced in the AC by a separate valve 20-gauge incision, and I/A was carried out until complete removal using balanced salt solution. Ease of removal was assessed. |
| 5 | 100 µL of this solution was substituted by 100 µL of $H_2O_2$ at the above-mentioned concentration or lactated Ringer's solution (control) through the first paracentesis using the Hamilton syringe. |
| 6 | The AC wash refilled with balanced salt solution. |
| 7 | The animals were kept under sedation for 5 hours and then killed with a lethal injection of pentobarbital. |
| 8 | The eyes were enucleated, and a 9 mm central corneal button was obtained with a trephine for determination of the extent of endothelial corneal damage. |
| 9 | The extent of endothelial damage was measured using the Janus green photometry absorbance technique. |

AC = anterior chamber; $H_2O_2$ = hydrogen peroxide; I/A = irrigation/aspiration

*Intraoperative Evaluation*

During surgery, each OVD was assessed for ease of injection and ease of removal using an arbitrary evaluation scale (1 = poor, 2 = good, 3 = excellent). Possible surgical complications were also recorded.

*Statistical Analysis*

Data of the percentage of endothelial cell lesion were tested for their normal distribution in each group. The measure of relevance, in this case the standardized difference and the related confidence interval, was used to compare the groups with graphical methods. For the measure of relevance, the Mann-Whitney statistics and the related confidence intervals were used to compare groups. A 2-sided 95% confidence interval was chosen in this explorative study design.

# Results

*Control Group (Lactated Ringer's Solution)*

Visiol exhibited a significant reduction in endothelial cell damage compared with the control (no OVD) ($P = .0045$), Healon ($P = .0089$), and Viscoat ($P = .003$) groups (Figure 1). There was an increase in endothelial cell damage in the Healon ($P = .0548$) and Viscoat ($P = .1828$) groups compared with the control group (no OVD).

*Peroxide Group (1 mM)*

A decrease in endothelial cell lesion was seen in all the OVDs tested compared with the control group in which no OVD was used (Figure 1). However, this reduction was statistically significant only in favor of Visiol ($P = .0337$). There was a tendency for endothelial cell lesion values to be lower in the Visiol group, than in the Healon ($P = .288$) and Viscoat ($P = .4688$) groups.

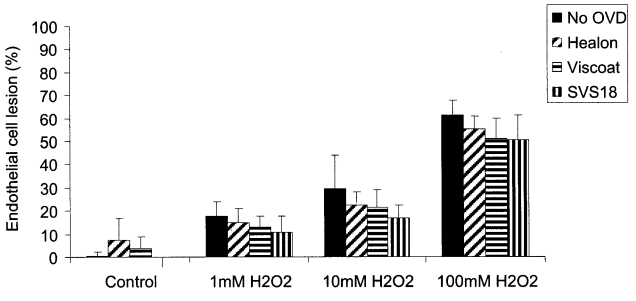


**Figure 1.** Overall results for endothelial cell lesion (mean ± SD, percentage) in the control (no OVD), Visiol, Healon, and Viscoat groups in presence of increasing concentrations of $H_2O_2$.

*Peroxide Group (10 mM)*

A significant decrease in endothelial cell lesion was observed in favor of the Visiol ($P = .0056$), Healon ($P = .0302$), and Viscoat ($P = .0336$) groups with respect to the control group (no OVD) (Figure 1). There was a strong trend in favor of Visiol to reduce more efficiently cell lesions of the endothelium than Healon ($P = .055$) and Viscoat ($P = .1013$).

*Peroxide Group (100 mM)*

The results in this group followed the same trend as those observed in the other groups. A significant decrease in the endothelial cell lesions was seen in favor of Visiol ($P = .0273$), Healon ($P = .0547$), and Viscoat ($P = .072$) compared with the control group (no OVD) (Figure 1). There was a tendency for values in the Visiol group to be a lower value than those in the Healon group ($P = .2176$) and equal than those in the Viscoat group ($P = .9118$).

*Intrasurgical Information*

No surgical complication was observed by the surgeon throughout the study. Healon was significantly ($P < .0001$) easier to inject (score of 3/3 or excellent), than Visiol and Viscoat (score of 1/3 or poor). There was no significant difference between Visiol and Viscoat (Figure 2). The ease of removal was judged to be excellent for Healon, good for Visiol, and poor for Viscoat (Figure 3). There was a significant ($P < .0001$) difference in favor of Healon versus Visiol and Viscoat and a significant ($P < .0001$) difference in favor of Visiol compared with Viscoat.

# Discussion

Different OVDs were challenged in the presence of increasing peroxide concentrations. Results showed

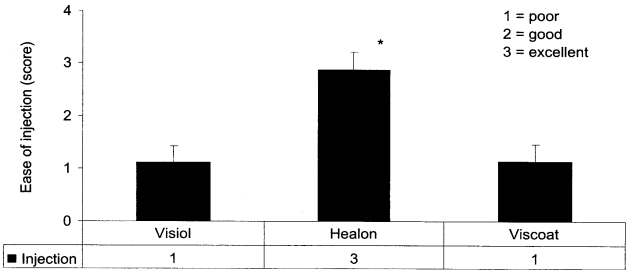


**Figure 2.** Ease of injection (mean score ± SD) of Visiol, Healon, and Viscoat. *$P < .0001$ versus Visiol and Viscoat.

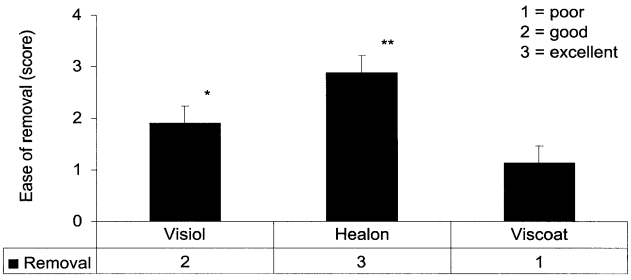


**Figure 3.** Ease of removal (mean score ± SD) of Visiol, Healon, and Viscoat. *$P < .0001$ versus Viscoat; **$P < .001$ versus Visiol and Viscoat.

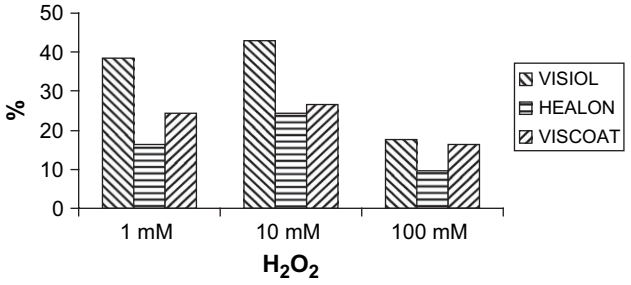


**Figure 4.** Overall results for reduction of endothelial cell lesion (percentage) in the control group (no OVD): Visiol, Healon, and Viscoat groups in presence of increasing concentrations of $H_2O_2$.

a dose-dependent increase in endothelial cell lesions in all the groups. All OVDs tested efficiently reduced lesions to the endothelium compared with the control group, in which no OVD was used. However, Visiol exhibited superior protective effects than Healon and Viscoat at all the peroxide concentrations tested, especially when we observe the mean reduction of endothelial cell damage (Figure 4). In the 1 mM peroxide group, Visiol provided a 38.6% reduction of endothelial cell damage compared with 16.2% for Healon and 24.4% for Viscoat. In the 10 mM peroxide group, this reduction was 43.1% for Visiol, 24.5% for Healon, and 26.5% for Viscoat. However, in the 100 mM peroxide group, we did not find such marked differences among the OVDs tested, and we think that the protective effect of the OVDs is overwhelmed by the oxidative stress caused by the peroxide at high concentration. This high concentration of peroxide is not comparable with concentrations observed in vivo during phacoemulsification, and we think that all the OVDs tested effectively protect the endothelium cells in standard, noncomplicated cataract surgery.

Our findings indicate that Visiol provides better endothelial protection against free radicals than Healon and Viscoat. The following sequence for the efficacy of endothelial cell protection can be established:

$$\text{Visiol} > \text{Viscoat} > \text{Healon} > \text{no OVD}.$$

The better endothelial cell protection of Visiol than with Healon and Viscoat may be attributed to the presence of mannitol in its formulation. In fact, mannitol is known to exert scavenging properties against free radicals, as shown by many authors.[5,8,9]

These results follow the same trend as those in previous studies in which the same model and method of evaluation were used.[4] This confirms the validity of the model used and the protective properties of SH against endothelial cell lesion caused by free radicals.

In a recent study, Augustin and Dick[10] confirmed the production of free radicals during phacoemulsification and the capability of OVDs (SH 1% better than hydroxypropyl methylcellulose 2%) to act as scavengers of free radicals. These facts, together with our results, strongly suggest the antioxidant properties of SH. Moreover, hyaluronate-binding sites on the corneal endothelium have been identified, suggesting that there should be a chemical action apart from the single mechanical protective effect.[11]

Other studies have shown a nonspecific beneficial effect of irrigating solutions containing several free radical scavengers, such as glutathione[12,13] or ascorbic acid,[14] in reducing endothelial cell loss secondary to ultrasound energy during phacoemulsification. All these substances were used in the phacoemulsification irrigating solution, but this is to our knowledge the first study in which the free-radical scavenger is included in the OVD. We hypothesize that the combination of sodium hyaluronate and a free-radical scavenger multiplies the protective effect on the corneal endothelial cells.

In conclusion, we showed that OVDs containing SH efficiently protected the endothelial cells against free radicals in an experimental model. The OVDs with higher concentrations of SH (Visiol and Viscoat) achieved better endothelial protection. Visiol showed to be the best OVD to protect endothelial cells against free-radical damage, probably due to its content of mannitol. Futher clinical studies will be necessary to confirm the benefit of mannitol in OVDs.

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