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Antioxidant status in rabbit aqueous humor after instillation of ascorbyl laurate-based nanostructures

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Abstract

Background: The aim of this work was evaluate the antioxidant effect of ascorbyl laurate (ASC12) based nanostructures applied topically to the cornea of ocular normotensive and hypertensive rabbits. The ASC12 was chosen for its capacity to form liquid lyotropic crystal and keeps its free radical trapping power.

Methods: The hypertension model was performed in six rabbits and was obtained by the application of intracameral injections of alpha-chymotrypsin in the right eye. A single 50 mL dose of ascorbyl laurate coagel 2% w/v (COA-ASC12) was applied topically to the cornea of six normotensive and six hypertensive rabbits. The aqueous humor samples were obtained before and after instillation of COA-ASC12 at different times (2h and 4h). Antioxidant capacity was determined via the reduction reaction with iron and tripyridyltriazine (FRAP) and the total proteins were measured using the Bradford reagent.

Results: The kinetic antioxidant capacity in the aqueous humor of normotensive and hypertensive rabbits showed a maxim increment at 4 hours instillation. Also, the antioxidant capacity in the aqueous humor of hypertensive rabbits was ten times lower than in normotensive rabbits.

Conclusion: This type of nanostructures has the potential to significantly improve the topical formulation for the prophylaxis and treatment of several eye diseases.

Keywords

nanostructures, ascorbyl laurate, antioxidant, glaucoma

Introduction

The eye is an organ continuously exposed to ionizing radiation, pollutants, industrial smoke and conduction fumes, which makes the eyeballs very susceptible to oxidative attack. This is the product of an imbalance between the production of free radicals (RL) and the elimination of these by means of the antioxidant defenses commonly known as oxidative stress (EO) [1].

To minimize oxidative stress, ocular tissues utilize a range of antioxidant defense systems, which include non-enzymatic and enzymatic antioxidants, however, as we age our antioxidant defense systems are overwhelmed, resulting in increased oxidative stress and damage to the tissues of the eye.

Ascorbic acid (AA), commonly called vitamin C (VC), is considered one of the most potent antioxidant agents in the body and it is present in the human aqueous humor at a concentration of 1 mM. Although it is present in a wide variety of tissues, the corneal epithelium has the highest concentration of AA, indicating that this antioxidant is particularly important to protect this tissue from oxidative stress [2].

According to the current literature it is likely that an imbalance between pro-oxidative factors and antioxidant capacity has been shown to play an important role in the pathogenesis of glaucoma and many of the markers of oxidative stress have been reported in glaucomatous disease [3,4].

According to the WHO, glaucoma is the second leading cause of blindness in the world; it is a progressive optic neuropathy, often caused by elevated intraocular pressure (IOP) due to high abnormal resistance to the drainage of the aqueous humor through the trabecular meshwork and Schlemm's canal [5]. Normal regulation of IOP occurs chiefly through the regulation of the volume of the aqueous humor in the anterior chamber of the eye. This process depends on the balance of a complex mechanisms involved in the production and output of the aqueous humor, including the stability and survival of the cellular phenotypes involved in this process and the correct maintenance of homeostasis [6,7]. If ocular hypertension (OHT) persists for a long time, it causes irreversible damage to all ocular structures, resulting in a characteristic decrease in the visual field that concludes with optic nerve atrophy and subsequent blindness [8].

Glaucoma is usually treated with hypotensive agents and therefore the efficacy of these systems depends on the pharmacodynamics of the hypotensor and the ability of the drug to permeate the cornea and reach its site of action in time and form (pharmacokinetics).

Topical administration of liquid dosage forms such as solutions is the most common strategy for treating ocular diseases, however it is well known that very fluid liquid formulations present several drawbacks to drug bioavailability, owing mainly to efficient eye mechanisms for drug removal from ocular surface. The development of more convenient pharmaceutical formulations becomes necessary in order to overcome this kind of limitation.

In this context, the use of surfactants in pharmaceutical technology has been extensively explored. Depending on the chemical structure, concentration and temperature, surfactants are able to form supramolecular aggregates with particular properties according to the concentration increases. In these cases, interactions between adjacent structures increase and consequently the system can revert to bigger and more complex structures, usually called liquid crystals (CLs). CLs formed through interactions between the surfactant and the solvent are known as lyotropic liquid crystals (CLLs).

They also retain the ability of AA to remove radicals, and their antioxidant effectiveness is comparable to other natural reducing agents, such as carotenes, polyphenols, and tocopherols.

Our research group has explored the use of these derivatives as carrier systems without deepening the "per se" effect that this type of system may have in modifying the antioxidant capacity of aqueous humor [9].

Although it is well established that AA is important for protecting the ocular tissues from oxidative stress, there is no evidence of formulations of VC that can penetrate membranes to protect internal ocular structures that can be damaged in the glaucoma pathology.

It is thus interesting to explore its permeation inside the eye and its potential effects on the antioxidant capacity of aqueous humor in normotensive animals and OHT models.

Materials and methods

Sample preparations

(The samples were prepared and characterized as described by *Palma et al*. 2006). 2% w/v aqueous suspensions (using isotonic and sterile dextrose solution (5 % w/v) as medium) of ASC12 were heated to 55°C to produce a self-assembly at a temperature higher than their critical micellar temperature (52.1 °C). The samples were then cooled to room temperature to obtain coagel (COA-ASC12) The ASC12 has been selected for previous results already published [9,10].

Animals

Twelve New Zealand rabbits (IOP average=11.39±0.92 mmHg) weighing 2–2.5kg were used. The rabbits were provided with food and water *ad libitum* in a temperature-controlled room (21°C±5°C) and exposed to 12h light: 12h dark cycles. In all cases, the appropriate measures were taken to minimize the discomfort and pain of the animals. The experiments were carried out following the CICC guidelines (FCM-UNC), coinciding with the Guidelines for the care and use of laboratory animals published by the National Institute of Health (NIH). This project has been approved by CICUAL - FCM - UNC (No. 44/2017).

Hypertension model

The OHT model was performed in six normotensive rabbits to simulate the characteristic conditions of the glaucoma disease. This model was obtained by the application of intracameral injections of alpha-chymotrypsin in the right eye (OD) [11].

Surgical technique: pilocarpine 2% w/v was instilled in the eye to cause miosis, then a paracentesis was performed and 0.1 ml of alpha chymotrypsin at a concentration of 3 mg/ml was injected into the anterior chamber using a 27-gauge cannula. After 3 minutes, the eye was washed with physiological solution and topical antibiotic and nonsteroidal anti-inflammatory were applied. A volume of 0.1 ml of physiological solution was injected into the anterior chamber of the left eye, which was used as surgical control. The IOP values were determined before and after induction of OHT, every 5 days for 40 days with digital veterinary manual tonometer (ICARE®). All animals were anesthetized with xylazine (0.2 ml/kg) and ketamine (0.8 ml/kg) before the surgical procedure.

Instillation of ascorbyl laurate based nanostructures in rabbit eyes

A single 50 mL dose of COA-ASC12 was instilled topically to the surface of eye in six normotensive and six hypertensive rabbits.

Determination of total antioxidant capacity

The aqueous humor samples (200 µL with TERUMO syringes) were obtained before and after instillation of COA-ASC12 at different times (2h and 4h). Upon completion of the examination, the animals were euthanized by CO_2 inhalation according to the protocol.

Antioxidant capacity was determined via the reduction reaction with iron and tripyridyltriazine (FRAP) and the total proteins were measured using the Bradford reagent. Ten µL of aqueous humor was used. 300 µL of a mixture of reagents (10 parts of 300 mM acetate buffer pH 3.6, 1 part of 10 mM (2,4,6-tripyridyl-s-triazine) TPTZ solution in 40 mM HCl and 1 part ferric chloride solution (FeCl3.6H2O) 20 mM were added). Aqueous solutions of known concentrations of Fe2 + (FeSO4.7H2O) were used as controls to perform the calibration curve and the final color was determined in a microplate reader (Bio-Rad) at 595 nm.

On the other hand, the determination of total proteins was carried out with the Bradford method. For this determination, 5 µl of sample was used, to which 250 µl of Bradford reagent was added and it was read spectrophotometrically at 595 nm.

Finally, the results were expressed as µM FeSO4 / mg of proteins [12,13].

Statistical analysis

Data was expressed as means and standard deviations. The results were analyzed using Student's t-test (individual times) and MANOVA (T. de Hotelling) to compare antioxidant capacity in normotensive rabbits and in OHT models, at a level of significance of $p < 0.05$.

Results

The figure 1 shows the antioxidant capacity of normotensive rabbits and OHT after and before the application of CLL. The antioxidant capacity of normotensive rabbits increased to a maximum of 3087 ± 45 μM $FeSO_4.6H_2O$/mg protein at 4 hours instillation. A significant increase in antioxidant capacity as a function of time was also observed in hypertensive rabbits (240 ± 8 μM $FeSO_4.6H_2O$/mg protein). The antioxidant capacity in the aqueous humor of hypertensive rabbits was ten times lower than in normotensive rabbits.

Insert Figure 1

Discussion

Numerous studies have shown that RL production and subsequent damage usually increase with age, and therefore plays an important role in pathologies that appear with aging. Advanced age is a risk factor for glaucoma, and IOP appears to increase with age. In consequence, patients with glaucoma are genetically more prone to free-radical damage. Although the most important pathological factor for the progression of glaucoma is IOP increment, there are other factors such as the reduction of antioxidant capacity and increased free radicals, which may also play an important role in the progression of glaucoma [14].

A balance between free radical production and antioxidant potential occurs under normal physiological conditions, and oxidative stress begins its deleterious effect at the same time to the antioxidant effect decreases. Altered antioxidant mechanisms and increased markers of oxidative stress such as DNA oxidation, protein carbonyl formation and lipid peroxidation has been documented in glaucoma patients.

The antioxidant status of a biological sample could be regarded as an indicator of oxidative stress: a decrease in the antioxidant capacity of body fluids may be the consequence of increased oxidative processes. We attempted to characterize the total antioxidant capacities of aqueous humor in normotensive and hypertensive rabbits. Our results suggest that hypertensive rabbits had significantly lower levels of antioxidant capacity in aqueous humor. Data from Ferreira et al, suggested that oxidative stress could have a role in the

pathogenesis of POAG and although their results were obtained in the aqueous humor of glaucoma patients, they coincide with ours [15].

Overall, these data indicates that altering the redox state contributes to the development of glaucoma, although the mechanisms by which oxidative stress triggers this event have not been fully elucidated.

Although the main pharmacological approach to glaucoma treatment is to decrease IOP, therapeutic interventions aiming to reduce in vivo oxidative stress seem to be useful in patients suffering from the disease. In spite of all reported experimental data, there is still incomplete knowledge with which to understand whether free radical generation is a primary or a secondary event in glaucomatous neurodegeneration.

Vitamin C due to its aqueous solubility can only be used topically for superficial ocular lesions. Amphiphilic derivatives of ascorbic acid-vitamin C (ASCn) have demonstrated the ability to form supramolecular aggregates resembling CLL and improve the lipids solubility and therefore improves the permeability through the ocular membranes, and can be used as a possible tool of ocular neuroprotection for pathologies such as glaucoma [9].

This derivative, which is capable of forming lamellar CLL, according to previous results, was able to modify the antioxidant capacity of the aqueous humor when was topically applied to the eye surface. An increase of antioxidant capacity was observed in normotensive and hypertensive rabbits which could be attributed to an increase in the concentrations of AA (such as laurate) in the anterior chamber. This behavior would be indicative of the potential utility of ASC12 in the treatment or prophylaxis of several ocular diseases, including glaucoma.

An effective synergistic effect is expected through the incorporation of antiglaucomatous drugs into this drug delivery system. COA-ASC12 has also demonstrated drug permeation enhancement when administered onto ocular mucosa [9,16]. This strategy is being evaluated in our working group.

Future Perspective

The current treatment of glaucoma is focused only on decreasing the IOP. However, it is clear that the future perspective would be to complement

topical hypotensive treatment with a topical ocular neuroprotective treatment. The nanostructures presented here would become a smart choice in this field.

Executive summary

Glaucoma disease and current treatment

- Glaucoma is the second cause of global irreversible blindness.
- Conventional glaucoma treatment aims to lower IOP.
- The oxidative stress involved in glaucoma evolution is not currently treated.

Proposed delivery device

- ASC12, has the capacity to form CLL and keeps its free radical trapping power.
- The CLL of ASC12 were able to increase the antioxidant capacity in experimental rabbits
- This type of nanostructures could be used for a more efficient prophylaxis & treatment of several eye diseases.

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Figure 1: Antioxidant capacity determined by the FRAP method in aqueous humor of normotensive and OHT rabbits treated and untreated with COA-ASC12. Control values represent rabbits without treatment with COA-ASC12. The line with circles represents the antioxidant capacity of the normotensive rabbits while the line with triangles indicates the antioxidant capacity of the hypertensive rabbits. The mean and standard deviation values of antioxidant capacity, expressed in µM FeSO4.6H20/mg protein, were compared statistically with a significance level of $p < 0.05$.