ORIGINAL RESEARCH

The identification of uric acid as the major reducing agent on the corneal surface

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Abstract

Aim: To identify the compound accountable for the high reducing power lining the surface of the corneal epithelium.
Materials and methods: Chinoy's histochemical method was used to demonstrate the occurrence of a high reducing agent on the corneal surface. Ascorbic and uric acid concentration in the tear and cornea were determined by high-pressure liquid chromatography using a μ-Bondapak-NH_2 column eluted by 10 mM ammonium phosphate.
Results: A solid black stain was observed on the surface of a cornea after incubating a rabbit eye in a fixative with silver nitrate. Uric acid concentration in the epithelium was much higher than in the stroma and endothelium. A supersaturated uric acid concentration was observed in human tears. The concentration of uric acid was much higher than that of ascorbic acid. Mixing ascorbic acid with silver nitrate produced fine silver granules. Uric acid and silver nitrate formed coarse precipitate.
Conclusion: Uric acid is the major reducing agent on the surface of the cornea. Uric acid on the corneal surface may have a role in preventing oxidative damage by air.

Key words: *Antioxidant, Ascorbic acid, Cornea, Epithelium, Silver, Tear, Uric acid*

Introduction

The corneal epithelium is constantly subjected to free radical stress because of its close proximity to air. We have used Chinoy's method[1] to investigate the role of ascorbic acid as a physiological antioxidant in ocular tissues in previous studies.[2-4] Recently, we used Chinoy's method to study ascorbic acid in tears. Chinoy *et al.* described a simple histochemical method to visualize ascorbic acid distribution in tissue sections.[1] Their method involves fixing fresh tissues in an alcohol-acetic acid solution containing 5% silver nitrate. Ascorbic acid reduced silver nitrate to form fine silver granules. The location of silver granules in a tissue section indicates the location of endogenous ascorbic acid. The specificity of Chinoy's method for the visualization of ascorbic acid gained support from the observation that there were no silver granules in tissues of scorbutic animals. The addition of acetic acid to the silver nitrate ($AgNO_3$) solution was used to eliminate interference by major physiological reducing agents, including glutathione, sugar, and amino acids. These researchers cautioned about possible false positive results due to the interaction of silver nitrate with physiological compounds that have not yet been examined. The interaction between uric acid and silver nitrate was not tested in their study.

We employed Chinoy's method in previous studies to visualize the location of ascorbic acid in the ocular tissues of

chicken embryo[2,3] and rats.[4] A high density of silver granules was observed in the areas where ascorbic acid concentration was expected to be high. For example, dense silver granules in the vitreous cavity of the embryonic optic vesicle indicate the high amount of ascorbic acid transported into the optic vesicle during the expansion of embryonic chicken eye.[2] The high ascorbic acid concentration in the vitreous humor of chickens was confirmed by chromatographic analysis.[3] Ascorbic acid was very low in rat's intraocular fluid and negligible silver granules were noted in rat's anterior chamber and vitreous cavity. Ascorbic acid was confined to the outer nuclear layer of rat's retina.[4]

In our previous studies, we could not explain a solid black deposit on the surface of the corneal epithelium in chicken, rats, and rabbits. We have now collected evidence that the high amount of uric acid on the corneal surface accounts for the high reducing power of the corneal surface, causing the silver deposit upon interaction with silver nitrate.

Materials and methods

Silver nitrate staining for ascorbic acid

The staining solution consisted of 5 g $AgNO_3$ in 34 ml of water, 5 ml of glacial acetic acid, and 66 ml of ethanol.[1] The eyes were immersed in this solution overnight at 0-4°C in the dark. The unreacted $AgNO_3$ was removed by immersing the eyes in 100 ml of 5% sodium thiosulfate for 30 minutes. The eyes were fixed in 4% formalin and embedded in paraffin for sectioning. The counter stain was 1% methyl green.

Ascorbic acid and uric acid concentrations in tear, aqueous humor, and corneal tissues were analyzed by high pressure liquid chromatography using a µ-Bondapak-NH_2 column (0.9 x 30 cm) [Waters Associates, Medford, MA, USA]. The tissues were weighed and homogenized in 0.5 ml methanol. The coagulated proteins were removed by centrifuge. An aliquot of 5 µl of the supernatant fraction was injected into the column and eluted by 10 mM ammonium phosphate ($NH_4H_2PO_4$) at 1 ml/min as previously described.[4]

Tears were collected from healthy adults by absorption into polyester rods and recovered by centrifuge.[5] Specimens were taken at the Clinic/Vision Research Lab of Singapore Polytechnic. The tears were diluted 100-fold by 10 mM $NH_4H_2PO_4$ and 10 µl was analyzed by the chromatographic method described above.

Results

Reducing agent on the corneal surface detected by silver nitrate

Chinoy's method was useful for our previous study, to visualize the distribution of ascorbic acid in eye tissues.[2,4] Fine silver granules were observed in locations containing high concentrations of ascorbic acid. However, a solid black layer was observed on the corneal surface (**Figure 1**), much darker than any area in the eye. The same results have been observed on the corneal surface of chicken and rats.

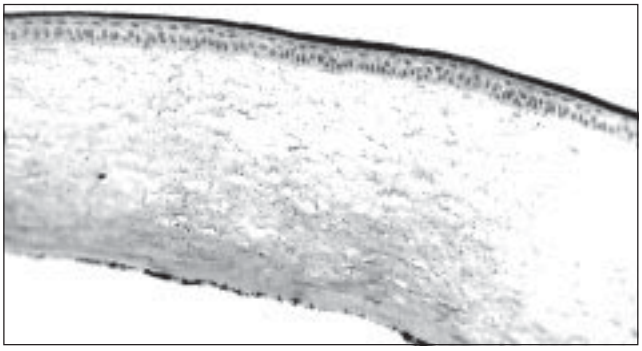

Figure 1. Reducing agent on a rabbit's corneal surface (x 620). A black deposit was observed on the surface of a rabbit cornea. The eye was incubated with silver nitrate, embedded, and sectioned.

Uric acid distribution in the cornea

Uric acid concentration was much higher in the corneal epithelium than in the stroma and endothelium (**Table 1**). There was a barely detectable amount of uric acid in the rabbit's aqueous humour.[6,7] Similar results were observed in both human and rabbit cornea. The tear film is included in the preparation of the epithelium.

Uric acid and ascorbic acid in human tear

Human tears were directly absorbed into a polyester rod and recovered by centrifuge as previously described.[5] Uric acid is a stable compound; the value of 15.7 mg/dl is a supersaturated concentration of uric acid in water (**Table 2**). Ascorbic acid is easily oxidized and the oxidized ascorbic acid is not detectable by ultraviolet. The amount of ascorbic acid in tears may be underestimated due to the unknown amount of oxidation prior to analysis (**Table 2**).

In vitro reaction of $AgNO_3$ with uric acid and ascorbic acid

An equal volume of $AgNO_3$ (5 g/dl) and ascorbic acid (20 mg/dl) were mixed in a test tube and placed on a microscope slide with a cover slip. Mixing ascorbic acid with silver nitrate produced fine granules, as shown in

Table 1. Uric acid concentration in different layers of the cornea.

	Number	Rabbit mg/100g tissue	Human mg/100g tissue
Epithelium	4	22.1 ± 10.9	33.4 ± 10.5
Stroma	4	5.2 ± 1.3	1.4 ± 0.5
Endothelium	4	7.0 ± 3.2	8.4 ± 1.7

Table 2. Uric and ascorbic acid concentration in human tears.

Number	Uric acid (mg/dl)	Ascorbic acid (mg/dl)
6	15.7 ± 3.5	1.9 ± 1.1

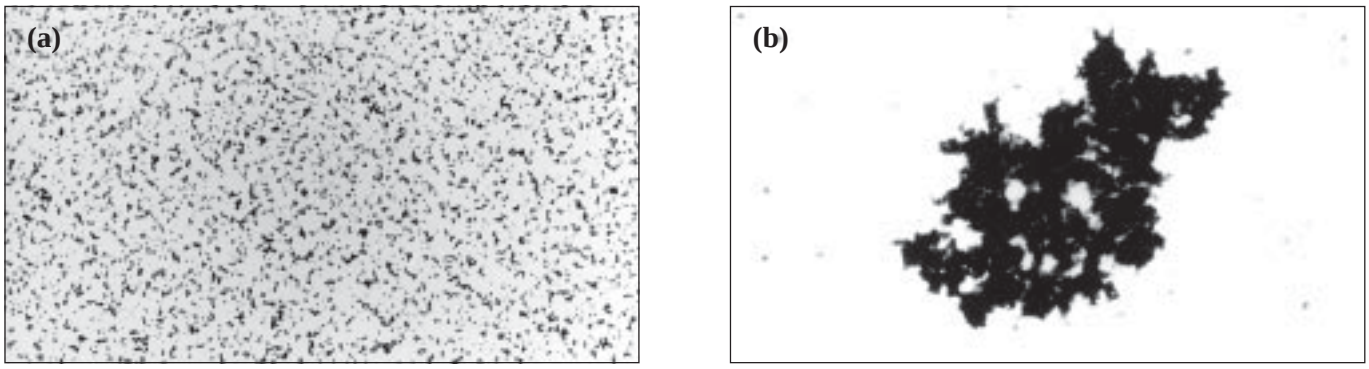

Figure 2. ***In vitro*** **interaction between silver nitrate and (a) ascorbic acid or (b) uric acid.**

Figure 2a. When $AgNO_3$ was mixed with uric acid (5 mg/dl), a coarse black precipitate formed (**Figure 2b**). The appearance of a solid black aggregate of uric acid-silver complex has an appearance similar to the solid dark stain on the corneal surface shown in **Figure 1**.

Discussion

These data demonstrate the occurrence of a strong reducing power on the corneal surface by staining with silver nitrate (**Figure 1**). We conclude that the solid black stain on the epithelium is the reaction product of silver with a high concentration of uric acid on the corneal surface. The high amount of uric acid on the epithelium was confirmed by chromatographic analysis of each corneal layer and the tears. The uric acid concentration in the epithelium is much higher than in the stroma and endothelium. A supersaturated concentration of uric acid was observed in tears. The corneal epithelium preparation included the tear film. Since the black stain was observed only on the surface of the epithelium, uric acid observed in the epithelium preparation is due to the occurrence of uric acid in the tear film on the surface of the epithelium.

The black deposit on the corneal surface cannot be the product of ascorbic acid and silver nitrate. The concentration of uric acid in the tears was much higher than that of ascorbic acid (**Table 2**). Furthermore, uric acid interacts with silver nitrate to form a coarse precipitate (**Figure 2b**) accountable for the solid black stain on the corneal surface. Ascorbic acid reduced silver nitrate to fine silver granules (**Figure 2a**), which have a very different appearance from the black stain on the corneal surface (**Figure 1**).

The corneal epithelium is constantly subjected to free radical stress because of its close proximity to air. A strong antioxidant is needed to prevent oxidative damage to the epithelium. The major water soluble antioxidants in living cells are glutathione, ascorbic acid, and uric acid. The antioxidant potency of glutathione and ascorbic acid rely on the intracellular enzymes that keep them in the reduced form.[8,9] In the extracellular space of corneal epithelium, both ascorbic acid and glutathione are irreversibly oxidized by air. The oxidized products do not have antioxidant value. The special feature of uric acid is its stability in air. It forms a complex with metallic ions to prevent metal catalyzed oxidation of physiological compounds.[10]

The high uric acid level on the corneal surface resembles that of the epithelial lining of human respiratory tract. Cross *et al.* observed a high concentration of uric acid in nasal secretions and in the fluid lining the epithelium of the human respiratory tract.[11] There was negligible ascorbic acid and glutathione observed in their specimens. They postulated that the role of uric acid on the epithelium is to prevent damage to the epithelium by a trace amount of ozone in inhaled air. Uric acid can be a common antioxidant to prevent ozone damage in all epithelial tissues exposed to air.

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