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Evaluation of histamine-induced conjunctival oedema in guinea pigs by means of image analysis

A Takahashi,¹ T Sumi,¹ K Tada,¹ H Mibu,² D Shii,² A Kayasuga,³ A Fukushima¹

¹Department of Ophthalmology and Visual Science, Kochi Medical School, Nankoku, Japan

²Santen Pharmaceutical Company, Osaka, Japan

³Cine-Science Laboratory Co, Ltd, Tokyo, Japan

Correspondence to

Dr Atsuki Fukushima,
Department of Ophthalmology and Visual Science, Kochi Medical School, Kohasu, Ogo-cho, Nankoku 783-8505, Japan;
fukushima@kochi-u.ac.jp

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ABSTRACT

Background Conjunctival oedema is commonly observed in patients with allergic conjunctivitis and can be induced by histamine. In animal models of allergic conjunctivitis, conjunctival oedema is generally evaluated by measuring the extravasation of Evans blue dye into the conjunctiva. A limitation of this method is that it only allows evaluation at a single time point. The aim of the present study was to investigate kinetic changes in histamine-induced bulbar oedema.

Methods Evans blue dye was injected intravenously into male guinea pigs. Histamine eye-drops were administered 30 min later. One group of animals received levocabastine (an antihistamine) eye-drops 10 min before histamine challenge. A digital camera was used to obtain images of the bulbar conjunctiva at 1 min intervals until 30 min after histamine challenge. The conjunctivas were then harvested, and the concentration of Evans blue was measured. The ImageJ software was used to analyse the images by counting the number of absolute pixel values.

Results The degree of conjunctival oedema increased progressively until 20 min after histamine challenge and then stabilised. Correspondingly, the number of absolute pixel values increased significantly until 5 min after histamine challenge, then increased gradually until the 20 min time point and finally plateaued. Pixel values were significantly lower in animals treated with levocabastine than in control animals. A significant correlation was observed between the pixel values of the conjunctival images and the concentration of Evans blue in the conjunctiva.

Conclusions This is the first study to have quantitatively evaluated kinetic changes in histamine-induced bulbar oedema by means of image analysis.

Conjunctival hyperaemia and oedema are commonly observed in patients with allergic conjunctivitis (AC).¹ Although grading scores are used to evaluate conjunctival hyperaemia and oedema in clinical practice,^{2–3} the evaluation is subjective and there is scope for inter-rater discrepancy. In a previous study, we demonstrated that image analysis can be used to quantitatively evaluate conjunctival hyperaemia that is induced by histamine⁴ or an allergic reaction.⁵ In this study, we demonstrated that conjunctival oedema develops several minutes after conjunctival hyperaemia and complicates its evaluation.

Clinical grading scores have also been used to evaluate conjunctival oedema in animal models of AC.^{6–7} An alternative method of evaluation is the intravenous injection of Evans blue dye before the induction of AC, with subsequent measurement of the concentration of extravasated dye in the

conjunctiva.^{8–10} Although this provides a quantitative evaluation of conjunctival oedema, it has 2 major limitations: (1) it is necessary to kill the animals to measure the concentration of the dye, and (2) it does not provide an evaluation of the kinetic changes involved in the development of conjunctival oedema. To overcome these limitations, we aimed to quantitatively evaluate conjunctival oedema by means of image analysis.

MATERIALS AND METHODS

Animals and induction of conjunctival oedema

A total of 14 male Hartley guinea pigs weighing 300–350 g were obtained from Kiwa Laboratory Animals Co Ltd (Wakayama, Japan). The animals had been bred in an animal facility of Cine-Science Laboratory Co, Ltd (Tokyo, Japan) under conventional conditions of temperature (23°C (±2)), relative humidity (60% (±10)) and light (12 h of light and 12 h of dark), and with free access to food and water. The 5-week-old animals were anaesthetised with 75 mg/kg urethane and then positioned under a microscope. To prevent drying of the surface of the eye and to maintain adequate space for observation, the right eye of each animal was fixed using a tape as described previously.^{4–5} To induce conjunctival oedema, 20 µl of 1000 µg/ml histamine (Wako, Osaka, Japan) was pipetted onto the surface of the eye. All procedures were carried out according to the guidelines of the Association for Research in Vision and Ophthalmology *Statement for the Use of Animals in Ophthalmic and Vision Research*.

Evaluation of conjunctival oedema

A digital camera (DXM 1200; Nikon, Tokyo, Japan) was used to record close-up images of the conjunctiva. RGB colour bitmap images (1280 × 1024 pixels) were taken at 1 min intervals beginning before histamine challenge and continuing until 30 min after histamine challenge. In a total of 14 animals, Evans blue (15 µg per animal; Tokyo Chemical Industry Co, Ltd, Tokyo, Japan) was injected intravenously 30 min before histamine challenge to achieve a more precise visualisation of the conjunctival oedema.

Treatment with levocabastine eye-drops

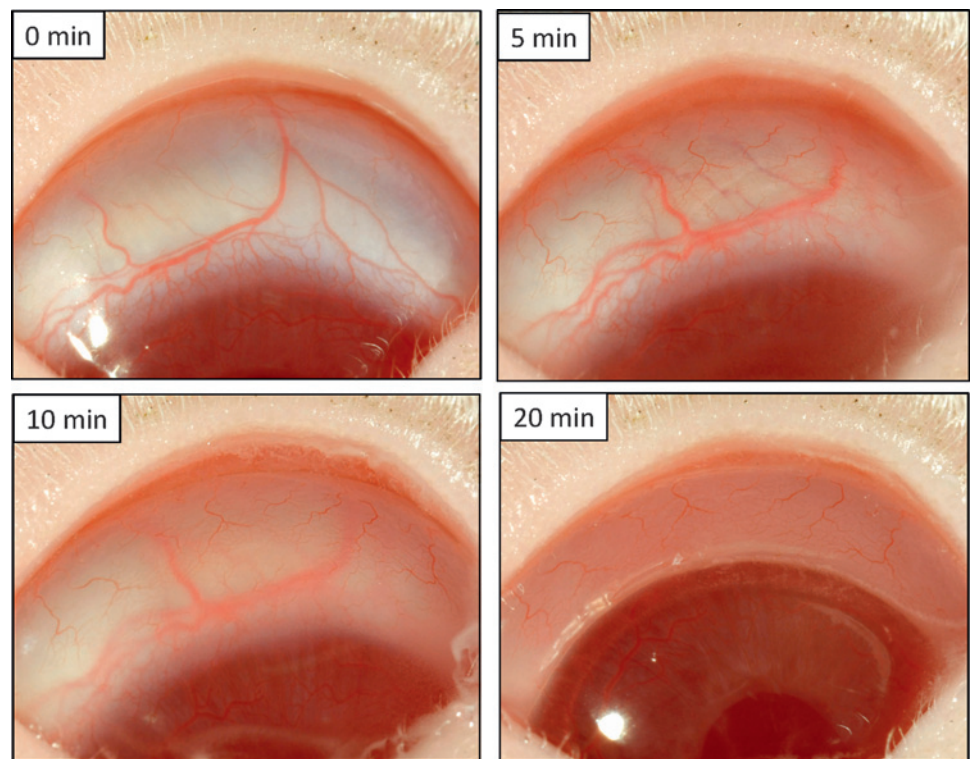
In a total of 7 animals, levocabastine eye-drops (10 µl per eye; Santen Pharmaceutical Co Ltd, Osaka, Japan) were administered 10 min before histamine challenge. No eye-drops were administered to the 7 control animals.

Image analysis

The ImageJ software (available at <http://rsb.info.nih.gov/ij/>), which is distributed by the National

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Figure 1 Kinetic changes of histamine-induced conjunctival oedema. Male guinea pigs were challenged with histamine eye-drops. A digital camera was used to obtain images of the conjunctiva at 1 min intervals until 30 min after histamine challenge; these were recorded as an RGB colour bitmap image. A representative of the 7 animals is shown. After the 20 min time point, no further change in the degree of conjunctival oedema was apparent.



Institutes of Health, was used for image analysis. To reduce image background noise, the RGB colour image was split and a median filter was applied. Because Evans blue is present in the conjunctival vessels and in the conjunctival tissues, 2 approaches were used: (1) evaluation of the entire conjunctiva including the vessels and (2) evaluation of the conjunctival tissues excluding the vessels. For the first approach, we extracted images from the red channel; for the second approach, we deleted images extracted from the blue channel (the vessel component) from the images

extracted from the red channel. The images were presented as a value of 256 for the 0-to-255 grey scale.

Measurement of the concentration of Evans blue in the conjunctiva

The conjunctivas were harvested 30 min after histamine challenge. Evans blue was extracted for 48 h by placing the conjunctivas in a 2 ml mixture of 0.5% Na_2SO_4 and acetone (3:7). After centrifugation, the absorbance (620 nm) of the

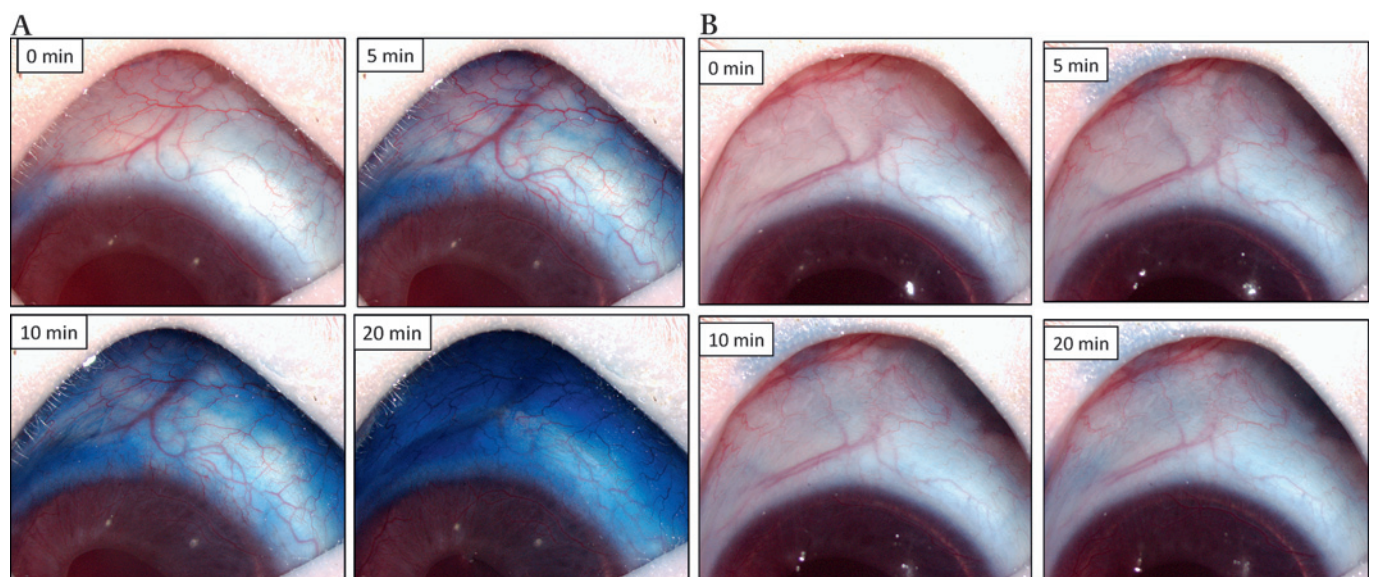


Figure 2 Visualisation of conjunctival oedema by means of Evans blue. Evans blue was injected intravenously into 14 animals 30 min before histamine challenge. A digital camera was used to obtain images of the conjunctiva at 1 min intervals until 30 min after histamine challenge, and these were recorded as described previously (A). Seven animals received levocabastine eye-drops 10 min before histamine challenge (B). A representative of the 7 animals in each group is shown. After the 20 min time point, no further change in the degree of conjunctival oedema was apparent.

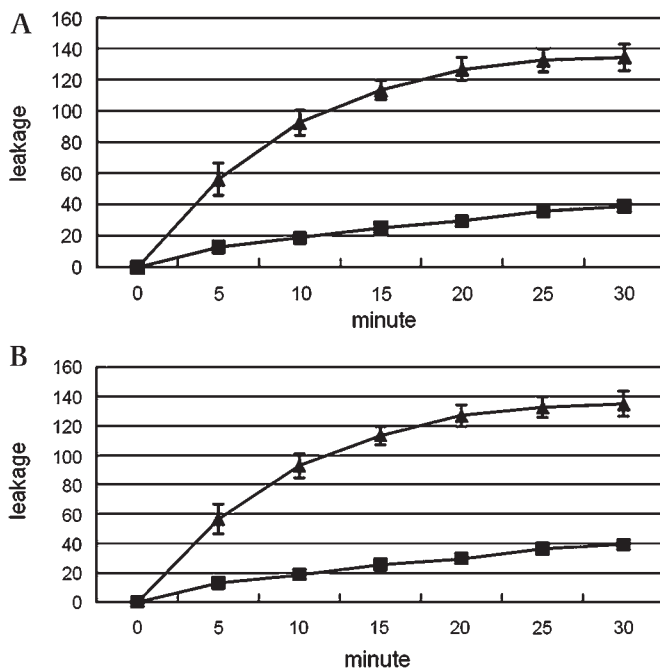


Figure 3 Evaluation of the extravasation of Evans blue into the conjunctiva by means of image analysis. Because Evans blue is present in the conjunctival tissues and the conjunctival vessels, 2 approaches were used to assess its extravasation by means of image analysis: (1) evaluation of the entire conjunctiva including the vessels (A) and (2) evaluation of conjunctival tissues excluding the vessels (B), as described in the Materials and Methods section. Significantly lower pixel values were observed in animals treated with levocabastine (closed square) than in control animals (closed triangle), irrespective of the method of evaluation used (A and B). There were 7 animals in each group. $p < 0.01$ at all time points later than 5 min after histamine challenge.

supernatant was determined using a spectrophotometer. The data are expressed as Evans blue content (nanogram) per eye.

Three-dimensional view of conjunctival oedema

To reveal the levels of oedema in specific areas of the conjunctiva, we evaluated the concentration of Evans blue in each specific area of the conjunctiva by means of the image analysis. The concentration of Evans blue is shown according to a scale ranging from 0 (white) to 255 (dark blue) as a z-axis.

Statistical analysis

Student *t* test was used to compare absolute pixel values between the levocabastine-treated and untreated groups. To examine the correlation between the pixel values obtained by image analysis and the concentration of Evans blue, the Spearman rank correlation coefficient by rank test was used. A level of $p < 0.05$ was accepted as statistically significant.

RESULTS

Kinetic changes in histamine-induced conjunctival oedema: digital colour images

In our previous study of histamine-induced conjunctival hyperaemia, we found that conjunctival oedema developed 5 min after histamine challenge. In the present study, we monitored kinetic changes by means of a digital camera. Images of the conjunctiva were obtained at 1 min intervals until 30 min after histamine challenge. Conjunctival oedema appeared 5 min after histamine challenge (figure 1). Conjunctival oedema

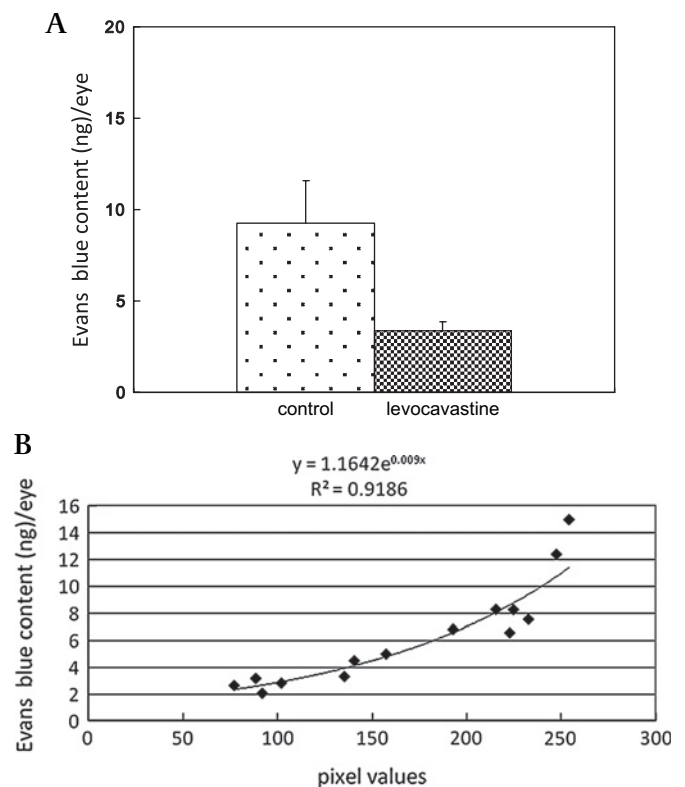


Figure 4 Correlation between image analysis and the concentration of Evans blue in the conjunctiva. The conjunctivas were harvested 30 min after histamine challenge, and the concentration of Evans blue was measured. Digital images of the conjunctivas had been obtained immediately before harvesting. (A) The concentration of Evans blue in the conjunctiva. Data are expressed as Evans blue content (nanogram) per eye. A significant difference was observed between the levocabastine group and the control group ($p < 0.001$). (B) Correlation between the pixel values obtained from image analysis and the concentration of Evans blue. Each dot represents 1 animal. A significant correlation was noted ($R^2 = 0.9186$).

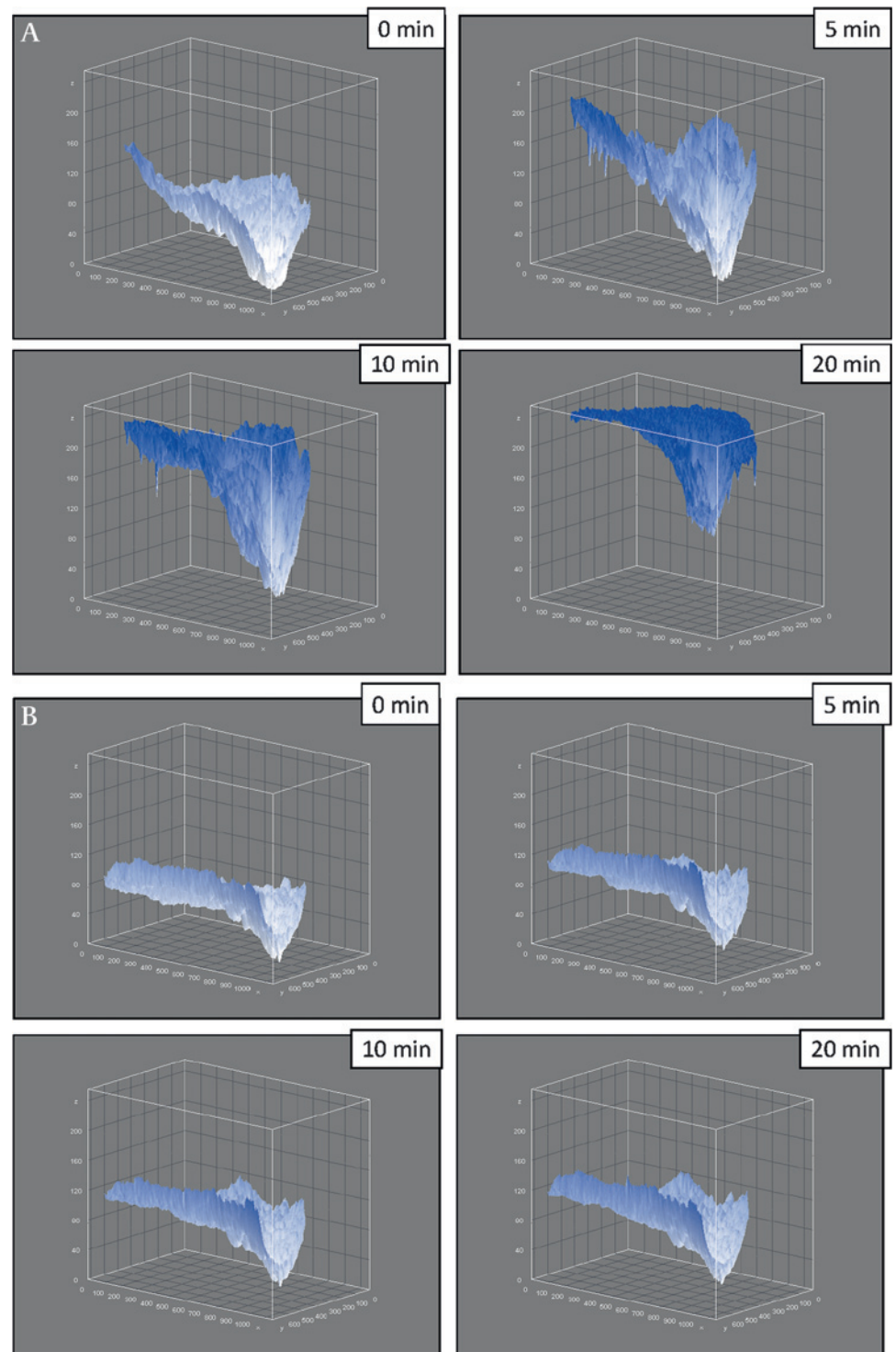
became more apparent 10 min after histamine challenge and continued to increase until the 20 min time point (figure 1). The degree of conjunctival oedema continued to increase between the 20 min and the 30 min time points, although no difference in the level of oedema between these 2 time points was clinically apparent (data not shown).

Kinetic changes of histamine-induced conjunctival oedema: evaluation according to the extravasation of Evans blue

To visualise the conjunctival oedema more precisely, Evans blue was injected intravenously into 7 animals 30 min before histamine challenge. Images of the conjunctiva were obtained at 1 min intervals until 30 min after histamine challenge. In most of the animals, similar to that in previous reports,^{11 12} a small amount of Evans blue was observed in the conjunctiva before histamine challenge (figure 2A). The concentration of Evans blue increased for 5 min after histamine challenge, and its distribution appeared to follow that of the vessels (figure 2A). The concentration of Evans blue in the conjunctiva increased gradually; by the 20 min time point, it was difficult to observe the conjunctival vessels because of the density of the extravasation (figure 2A). After the 20 min time point, the extravasation of Evans blue slowly increased until the 30 min time point, although this difference was not clinically apparent (data not shown). At all time points, the extravasation of Evans blue

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Figure 5 Distribution of the extravasation of Evans blue into the conjunctiva. To evaluate its extravasation into specific areas of the conjunctiva, the concentration of Evans blue was evaluated in each area of the conjunctiva. The data are presented as a 3-dimensional image. Ten minutes before histamine challenge, 7 animals were left untreated (A) and 7 animals received levocabastine (B). A representative of the 7 animals is shown. After the 20 min time point, no further changes in the 3-dimensional images were apparent.



appeared to be less in animals treated with levocabastine than in control animals (figure 2B).

Evaluation of the extravasation of Evans blue into the conjunctiva by means of image analysis

To provide a quantitative evaluation of the concentration of extravasated Evans blue, the digital images were analysed using National Institutes of Health Image. Because Evans blue is present in the conjunctival vessels and in the conjunctival tissues, 2 approaches were used: (1) evaluation of the entire conjunctiva including the vessels (figure 3A) and (2) evaluation of the conjunctival tissues excluding the vessels (figure 3B).

Irrespective of the approach used, a significant increase in the pixel values of Evans blue was first observed 5 min after histamine challenge (figures 3A,B). The extravasation of Evans blue increased steadily until the 20 min time point (figures 3A,B) and then plateaued (data not shown). The pixel values obtained for animals treated with levocabastine eye-drops were significantly lower than those obtained for control animals (figures 3A,B).

Reliability of image analysis

To confirm the reliability of image analysis as a quantitative method of evaluation, 14 animals were injected intravenously with Evans blue. Of these, 7 received levocabastine and 7 were

left untreated. All animals were then challenged with histamine eye-drops. The conjunctivas were harvested 30 min later, and the concentration of Evans blue was measured. Digital images had been obtained immediately before harvesting. Because we had found a strong correlation between the 2 methods of evaluation (figures 3A,B), we used the entire conjunctiva, including the vessels, for this evaluation. The pixel values (figure 3A) and the concentration of Evans blue (figure 4A) were significantly lower in animals treated with levocabastine than in control animals. A significant correlation was observed between the pixel values obtained by image analysis and the concentration of Evans blue (figure 4B).

Analysis of the distribution of extravasated Evans blue in the conjunctiva

As described previously, the extravasation of Evans blue into the conjunctiva was non-uniform (figure 2). To evaluate its extravasation into specific areas of the conjunctiva, we evaluated the concentration of Evans blue in all areas of the conjunctiva. The data are presented as a 3-dimensional image. The concentration of Evans blue is shown according to a scale ranging from 0 (white) to 255 (dark blue). Before histamine challenge, bright blue spots were noted in confined areas of the conjunctiva (figure 5A). Five minutes later, the bright blue spots had increased and dark blue spots were also observed (figure 5A). Ten minutes later, the dark blue spots increased, and 20 min later, dark blue was observed in all areas of the conjunctiva (figure 5A). In animals treated with levocabastine, bright blue spots were observed, but dark blue spots were barely detectable at any time point (figure 5B).

DISCUSSION

Conjunctival oedema is commonly observed in AC. However, its clinical evaluation is subjective rather than quantitative.^{2 3} Because no previous study has investigated the kinetic changes of histamine-induced conjunctival oedema, we aimed to evaluate this quantitatively.

Anterior-segment optical coherent tomography¹³ is a recent innovation that provides a convenient measure of the volume or the height (z-axis) of conjunctival oedema.^{14 15} However, this method only provides information concerning a single area and cannot be used to determine the total area (x-y-axis) of oedema in the conjunctiva. In the present study, we demonstrated that the area of conjunctival oedema can be evaluated quantitatively using a combination of image analysis and the intravenous administration of Evans blue. We observed a strong correlation between the pixel values obtained from the image analysis and the concentration of extravasated Evans blue in the conjunctiva. Thus, the image analysis presented here appears to be less prone to picking up background noise signals.

Although these findings provide strong evidence for reliability of the data with the correlation coefficient, there remains a question with regard to repeatability. Obviously, the degree of focus and the quality of images appear to have an impact on the results. These parameters were identical in this study. Furthermore, data that SEs are relatively small as shown in figure 3 indicate that variations among the animals are also relatively small. These facts suggest that the image analysis in this study can be repeatable though not complete.

Because image analysis is 2-dimensional, it cannot be used to determine the volume of conjunctival oedema (z-axis). To address this limitation, we evaluated the concentration of Evans blue in each area of the conjunctiva and presented the data as

a 3-dimensional image. This method allowed us to evaluate the entire conjunctiva and the volume of oedema in each specified area.

In the present study, we also examined whether image analysis could be used to evaluate the effects of antihistamine eye-drops on conjunctival oedema. Levocabastine, an antihistamine preparation, significantly suppressed histamine-induced conjunctival oedema, and we evaluated this quantitatively using image analysis. Although it is not yet possible to apply this method in clinical practice, dyes such as indocyanine green,¹⁶ which are approved for use in clinical settings, could be used to evaluate conjunctival oedema by means of the method described in this report. Alternatively, it may be possible to evaluate conjunctival oedema by means of a laser beam that specifically excites target serum protein in the conjunctiva. In any case, this technique would probably be of most use in a clinical research setting such as clinical trials to compare different topical anti-allergic eye-drops.

In conclusion, this is the first study to have quantitatively investigated the kinetic changes of histamine-induced conjunctival oedema.

Competing interests Declared. All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare financial support for the submitted work from Santen Pharmaceutical Company. All authors also declare that AF has received research grants and honorariums from Santen Pharmaceutical Company. No spouses, partners or children have relationships with commercial entities that might have an interest in the submitted work. There are no non-financial interests that may be relevant to the submitted work.

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