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Research Article

Safety and Stability of Tissue Plasminogen Activator Eye Drops

Kazumi Shinozaki[1]*, Ichiro Maruko[1], Sadao Hori[2] and Tomohiro Iida[1]

[1]Department of Ophthalmology, Tokyo Women's Medical University, Kawada-cho, Shinjuku-ku, Tokyo, Japan

[2]Department of Ophthalmology, Nishiarai Hospital, Nishiaraihon-cho, Adachi-ku, Tokyo Japan

***Corresponding Author:** Kazumi Shinozaki, Department of Ophthalmology, Tokyo Women's Medical University, Kawada-cho, Shinjuku-ku, Tokyo, Japan.

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Abstract

Purpose: Removal of the pseudomembrane for preventing pseudomembranous conjunctivitis progression is associated with pain and hemorrhage, and thus, a less invasive approach is needed. Tissue plasminogen activator (t-PA) solution, which is used to dissolve fibrin, may be feasible for non-invasive removal of the pseudomembrane in pseudomembranous conjunctivitis. This study aimed to investigate the safety and stability of the t-PA solution as eye drops in anticipation of clinical use for pseudomembranous conjunctivitis.

Methods: Two eye irritation tests of t-PA solution, namely, single- and multiple-dose instillation, were performed in 12 male Japanese white rabbits. The modified Draize criteria score was then compared between the test eye (i.e. t-PA administration) and control eye (i.e. saline administration). The blinking frequency during the single-dose eye irritation test was also compared. Next, the stability of frozen and refrigerated t-PA was evaluated. Samples were stored frozen for 4 or 11 months at -60°C or refrigerated for 1, 3, and 7 days. The biological activity of t-PA was then assessed after storage.

Results: There was no significant difference between the test eyes and control eyes ($P > 0.1$). There was no difference in the number of complete blinks in each group of treated eyes ($P > 0.1$). The t-PA activity was 96% and 82% after 4 and 11 months of frozen storage, respectively, while it was 99%, 104%, and 95% after 1, 3, and 7 days of refrigerator storage.

Conclusion: The t-PA eye drops is safe and stable and can thus be used for a less invasive removal of the pseudomembrane in pseudomembranous conjunctivitis.

Keywords: *Pseudomembranous Conjunctivitis; Tissue Plasminogen Activator; Eye Drop; Safety; Stability*

Abbreviation

t-PA: Tissue Plasminogen Activator

Introduction

Persistent or severe pseudomembranous conjunctivitis may cause symblepharon, conjunctival scarring, and corneal erosion. Although removal of the pseudomembrane under topical anesthesia is important to prevent progression of the condition, the procedure is often associated with pain and hemorrhage, thus causing patient anxiety. As such, some patients avoid this procedure. Furthermore, pseudomembranous conjunctivitis is more often observed in children, and this patient population is more likely to experience fear during procedures. Thus, a less invasive approach for the removal of pseudomembranes is needed.

Tissue plasminogen activator (t-PA) is a 527-amino molecule glycoprotein that plays an important role in the dissolution of fibrin. This biologically active substance with potent thrombolytic action is distributed in various tissues of the human body. The usefulness of t-PA in ophthalmology has been recently reported. New studies have shown that injection of t-PA into the anterior chamber and subconjunctiva is useful in reviving filtration after trabeculectomy [1-3]. Injection of t-PA into the anterior chamber or vitreous cavity also improved postoperative intraocular fibrin formation following cataract surgery or vitrectomy [4-8]. Further, t-PA has also been reported to be useful in patients with severe acute iridocyclitis and impending pupillary block glaucoma [9]. Moreover, there have been reports on the usefulness of subretinal injection of t-PA for hematoma removal [10-16]. Collectively, these studies indicate that the ophthalmic use of t-PA is relatively safe and effective.

As pseudomembranes are composed of fibrin, inflammatory cells, exudate, and conjunctival epithelium, t-PA, which can dissolve fibrin, as eye drops has a feasibility of being a potentially minimally invasive approach for the debridement of pseudomembranes.

Aim of the Study

Thus, this study aimed to examine the safety of t-PA for the debridement of pseudomembranes. Towards this goal, the t-PA solution was diluted for use as eye drops, and eye irritation tests were performed in rabbits. Stability after storage was also evaluated.

Materials and Methods

Ethics

This study was approved by the appropriate institutional review board. All animals in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Single-dose eye irritation test

Twelve male Japanese white rabbits weighing 2.27 - 2.73 kg were assigned to three groups involving 4 animals per group. Each group received 200,000 IU/ml (345 µg/ml) t-PA, 600,000 IU/ml t-PA, or physiological saline. The t-PA formulation used was a recombinant product (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan). The 200,000 IU/ml dilution of t-PA was the same as that reported for injection into the anterior chamber or vitreous cavity. The dilutions were prepared using sterile water for injection into the t-PA product kit. Physiological saline was used as the control. Eye drops (100 µl) were instilled into one eye of each animal using a micro pipetting system (Pipetman, Gilson, Inc., Wisconsin, USA), while the other eye was left untreated. The eye irritation level was scored pre-instillation; at 15, 30, and 60 min post-instillation; and at 24 h post-instillation based on the modified Draize method (Table 1) [17].

Cornea
A) Opacity
0 = No opacity (normal)
+1 = Scattered or diffuse areas of opacity (details of iris clearly visible)
+2 = Easily discernible translucent areas of opacity (details of iris slightly obscured)
+3 = Areas of opacity, no details of iris visible, size of pupil barely discernible
+4 = Complete corneal opacity, iris not discernible
B) Size of opacity
+1 = More than 0 and up to 1/4 of cornea
+2 = More than 1/4 and up to 1/2 of cornea
+3 = More than 1/2 and up to 3/4 of cornea
+4 = More than 3/4 and up to entirety of cornea

Iris
0 = Normal
+1 = Abnormal deepened folds, congestion, swelling, circumcorneal injection (any or a combination of any thereof), iris still reacting to light (sluggish reaction is positive)
+2 = No reaction to light, hemorrhage, gross destruction (any or all of these)
Conjunctiva
A) Redness of palpebral conjunctiva
0 = No congestion
+0.5 = Mucous membranes with very slight flush, slight dilation of perilimbal vessels
+1 = Definite congestion above normal, mucous membranes with definite flush, definite dilation of perilimbal vessels
+2 = Mucous membranes with marked flush, marked dilation of perilimbal vessels
+3 = Diffuse beefy red
B) Chemosis of palpebral conjunctiva
0 = No swelling
+0.5 = Swelling tendency of conjunctiva
+1 = Any swelling above normal
+2 = Obvious swelling with partial eversion of lids
+3 = Swelling with lids about half closed
+4 = Swelling with lids about half closed to completely closed
C) Redness of bulbar conjunctiva
0 = No congestion
+0.5 = Slight dilation of pericorneal vessels
+1 = Definite dilation of vessels
+2 = Marked dilation of vessels running toward palpebral conjunctiva or marked congestion
D) State of nictitating membrane
0 = No congestion
+0.5 = Dilating tendency of vessels and swelling tendency of membranes
+1 = Definite dilation of vessels, perilimbal congestion
+2 = Marked dilation of vessels, congestion of membranes
E) Discharge
0 = No discharge
+1 = Abnormal discharge increase (does not include small amount observed in inner canthus of normal eyes)
+2 = Discharge with moistening of the lids and hairs just adjacent to the lids

Table 1: *Modified Draize criteria (ocular irritation scoring system).*

The scores for individual eye irritation parameters at each time point were compared between the treated and untreated eyes in each animal. Statistical analyses were performed using the Kruskal-Wallis test. The number of complete blinks in the treated eyes 1 minute after instillation was counted and compared among the groups using one-way analysis of variance.

Multiple-dose eye irritation test

To examine the safety of multiple doses of t-PA solution, 12 male white Japanese White rabbits weighing 2.62 - 3.05 kg were similarly assigned to 3 groups. In each animal, 50 µl of the drug was instilled every 30 min for 10 times into one eye. The other eye was left untreated. The eye irritation level was assessed pre-instillation and at 1, 2, and 4h after the final instillation according to the modified Draize method [17]. Scores for individual eye irritation parameters at every time point were compared between the treated and untreated eyes in each animal using the Kruskal-Wallis test.

Stability of the t-PA solution

The recombinant t-PA product was reconstituted with sterile water included in the product for injection and further diluted with physiological saline to 200,000 IU/ml. Samples of the t-PA solution were then stored frozen at -80°C for 4 and 11 months, after which three aliquots of each were taken to check the stability of the solution. Three aliquots were also checked in the same manner as above after 1, 3, and 7 days of refrigerated storage. The buffer solution was used as a control. The biological activity of the t-PA solution was determined using S-2288 (Batch no. 132RFW; Daiichi Pure Chemicals Co., Ltd., Tokyo), a synthetic substrate specific for t-PA.

First, 100 µl buffer solution (consisting of 0.1 M Tris-HCl and 0.106 M NaCl, pH 8.4), 100 µl sample t-PA solution, and 100 µl substrate solution diluted in water (10 mM S-2288) was injected into a 96-well plate (IWAKI, 3860-096, Tokyo Japan). Next, absorbance was measured at 405 nm using a microplate reader immediately after the solutions were injected into the plate and at 5 min after the mixture was left to stand at room temperature. The change in absorbance per minute (ΔA/min) was calculated for each sample. To screen for bacterial contamination, bacterial cultures were performed in three samples frozen at -80°C for 4 months and 11 months and in three samples refrigerated at 4°C for 1 week.

Results

Single- and multiple dose eye irritation tests

Although a slight degree of redness in the conjunctival layer of the eyelids was observed in all three groups, no other symptoms of eye irritation were noted at any time point. In addition, there were no significant differences in eye irritation parameters. Table 2 shows the results of eye irritation assessment after instillation of a single dose of t-PA solution. The mean number of complete blinks in the treated eye at 1 minute after t-PA instillation was 1.25 ± 0.48/min in the t-PA-200,000 IU/ml group, 1.00 ± 0.58/min in the t-PA-600,000 IU/ml group, and 1.25 ± 0.75/min in the control group (Table 3), with no significant differences (P > 0.1). Moreover, there was no evidence of eye irritation in any of the treatment groups after instillation of multiple doses of t-PA solution.

			Time after instillation				
			Pre	15 min	30 min	60 min	24 hrs
Cornea	Opacity	Physiological saline	0	0	0	0	0
		200,000 IU/ml	0	0	0	0	0
		600,000 IU/ml	0	0	0	0	0
	Size of opacity	Physiological saline	0	0	0	0	0
		200,000 IU/ml	0	0	0	0	0
		600,000 IU/ml	0	0	0	0	0
Iris		Physiological saline	0	0	0	0	0
		200,000 IU/ml	0	0	0	0	0
		600,000 IU/ml	0	0	0	0	0

Conjunctiva	Redness of palpebral conjunctiva	Physiological saline	0.13	0.13	0	0.25	0
		200,000 IU/ml	0	0.13	0.13	0.13	0
		600,000 IU/ml	0.13	0.13	0.13	0.13	0
	Chemosis of palpebral conjunctiva	Physiological saline	0	0	0	0	0
		200,000 IU/ml	0	0	0	0	0
		600,000 IU/ml	0	0	0	0	0
	Redness of bulbar conjunctiva	Physiological saline	0	0	0	0	0
		200,000 IU/ml	0	0	0	0	0
		600,000 IU/ml	0	0	0	0	0
	State of nictitating membrane	Physiological saline	0	0	0	0	0
		200,000 IU/ml	0	0	0	0	0
		600,000 IU/ml	0	0	0	0	0
	Discharge	Physiological saline	0	0	0	0	0
		200,000 IU/ml	0	0	0	0	0
		600,000 IU/ml	0	0	0	0	0

Table 2: *Mean eye irritation scores after single-dose instillation (n = 4 rabbits/group). Data are presented as the mean.*

	Frequency/min
Physiological saline	1.25 ± 0.48
200,000 IU/ml t-PA	1.25 ± 0.75
600,000 IU/ml t-PA	1.00 ± 0.58

Table 3: *Blinking frequency after instillation of t-PA solution (n=4 rabbits/group). Data are presented as the mean ± SD.*

Stability

The mean activities of t-PA solution from the 3 sample aliquots were 100.00 ± 2.10 immediately after dilution, 95.96 ± 1.91 after 4 months of storage, and 81.53 ± 3.15 after 11 months of storage (Table 4). Meanwhile, in refrigerated solutions, the mean activities were 100 ± 4.41% immediately after preparation of the solution and 98.98 ± 4.49%, 103.60 ± 1.77% and 95.13 ± 3.91% after 1, 3, and 7 days of storage in the refrigerator, respectively (Table 5). All samples tested were negative for bacterial contamination.

Storage period	% of fresh sample
Immediately after preparation	100.00 ± 2.10
4 months	95.96 ± 1.91
11 months	81.53 ± 3.15

Table 4: *Activity change after freezing of t-PA solution (200,000 IU/ml, n = 3 samples). Data are presented as the mean ± SD.*

Storage period	% of fresh sample
Immediately after preparation	100.00 ± 4.41
1 day	98.98 ± 4.49
3 days	103.60 ± 1.77
7 days	95.13 ± 3.91

Table 5: *Activity change after cold storage (200,000 IU/ml, n = 3 samples). Data are presented as the mean ± SD.*

Discussion

Previous reports of ophthalmic t-PA administration to the subconjunctiva [2,3], anterior chamber [1,4-9], vitreous [8] and subretina [10-16] have demonstrated that it is reasonably safe for intraocular tissues. However, the safety and stability of t-PA eye drops have not been studied in detail. In this study, both single- and multiple-dose administrations of t-PA solution did not cause marked eye irritation in rabbits. Thus, in addition to a single-dose t-PA instillation, multiple-dose instillations may also be safe for use in ocular surface diseases. Further, histological assessment showed that the t-PA solution instillation did not cause any inflammatory changes, suggesting that t-PA instillation does not induce inflammatory or histological changes.

In a previous study using rabbits, 38% had detectable t-PA levels in the aqueous solution after a single t-PA drop instillation, and t-PA was detected in the anterior chamber in 75% of the rabbits after multiple t-PA drop instillation [18]. However, another study indicated that topical instillation or subconjunctival injection of the t-PA solution is ineffective for removing fibrin clots in the anterior chamber. Collectively, these results indicate that t-PA levels do not reach therapeutic levels in aqueous media [19,20]. This characteristic, however, is probably desirable for use in ocular surface diseases because poor intraocular penetration can localize the drug effect to the instillation site and improve drug safety. With respect to stability of the solution, Ward., *et al.* previously reported that t-PA should not be diluted in a balanced salt solution and stored at either room temperature or -20°C because precipitates form [21]. In contrast, Jaffe., *et al*. showed that the activity of 250 µg/ml t-PA solution was preserved even after 1 year of storage at -70°C, without bacterial contamination [22]. Our findings revealed that t-PA activity decreased by approximately 5% and 20% after 4 and 11 months of freezer storage at -80°C, respectively. Meanwhile, at refrigerator storage, t-PA activity decreased by approximately 5% after 1 week. No precipitate formation was observed in either storage method. These results indicate that the activity of the t-PA solution after dilution is well preserved for several months when stored at -80°C and for 1 week when stored under refrigeration. In addition to stable activity after storage, no contamination was observed. The excellent stability of the t-PA solution even after long-term storage at -70°C to -80°C and at low concentrations might also be advantageous from the point of view of cost effectiveness as this means that large amounts of eye drops can be prepared from a single commercial formulation.

Conclusion

The t-PA eye drops is safe and stable. Its instillation is useful for the non-invasive removal of the pseudomembrane in pseudomembranous conjunctivitis.

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Conflict of Interest

Dr. Shinozaki reports personal fees from Senju Pharmaceutical Co., Otuska Pharmaceutical Co., Novartis, outside the submitted work.

Dr. Hori has nothing to disclose.

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