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Examination of α-terpinene on Primary Eye Irritancy and Skin Sensitization

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Abstract

α-Terpinene has been known as a repellent against the mosquito *Culex pipiens pallens* Coquillett based on a human forearm bioassay. α-Terpinene showed significantly greater repellency than a commercial formulation, *N*, *N*-diethyl-*m*-methylbenzamide (deet). In this study, skin and eye sensitivity of α-terpinene (2%) was examined with bioassays using white New Zealand rabbits. There were somewhat gross and histological changes observed in these treatments. Eye irritancy assays examined gross changes to cornea, iris and conjuctiva, and histological changes to smear of ocular discharge and eye tissue. Treated rabbits were divided into two cohorts, a saline washed cohort (W) or a non-washed cohort (NW). Opacity of cornea and redness, chemosis and discharge of conjuctiva were observed in both cohorts, but disappeared within 4 and 10 days in W and NW, respectively. Main components of ocular discharges were fibrin, epithelial or epitheloid cells, lymphoid cells, erythrocytes and granulocytes. These abnormal cellular components disappeared within 4 days and 10 days in W and NW, respectively. No permanent histological differences were observed between the two cohorts. However, severe irritation was determined as 57.2 of I.I.O.I value on the first day after treatment. These findings indicate a spray-type solution containing 2% α-terpinene may serve as an alternative mosquito repellent and further studies need to reduce the eye irritation with formulation changes.

Keywords: α-Terpinene, Mosquito repellent, *Culex pipiens pallens*, Skin irritation, Eye irritation

Mosquitoes are vectors of a number of major diseases, such as malaria, dengue, yellow fever, and numerous types of encephalitis. Individually and collectively these diseases contribute significantly to worldwide morbidity and mortality of humans. The World Health Organization reported that in 2002 over 300 million people were infected with malaria alone, resulting in an estimated one million deaths[1]. About 90% of the deaths are in children aged under 5 years. In addition to transmitting diseases, mosquitoes are also major vectors throughout many temperate and tropical areas.

Control of mosquito vectors is becoming increasingly difficult because of resistance to insecticides[2,3]. In view of effective pesticides, repellents can be effective alternatives for protecting humans from mosquito-transmitted diseases or annoying mosquito bites. One repellent applied to exposed skin and widely used today is *N*, *N*-diethyl-*m*-methylbenzamide (deet). However, there are reports deet may be somewhat toxic because of high skin absorption after topical treatment[4,5]. Therefore, there may be a need to find other compounds as alternatives to deet and other currently used repellents.

Natural products derived from various botanical sources have been shown to be effective as repellents against mosquitoes[6,7]. Many natural products, such as essential oils and monoterpenes, are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) and used as fragrances in cosmetics, food additives, household products, medicine and insecticides.

Previous work, we showed four essential oils from different botanical sources to have potential mosquito repellent activity against *Culex pipiens pallens* Coquillett when applied to the skin of hairless mice[8,9]. Among the essential oils, that of thyme (*Thymus vulgaris*) proved to be the most effective by inhibiting mosquito bites at 91% to that of control at the concentration of 0.05% topical treatment. Thyme essential oil significantly extended the duration of protection until 3 bites by mosquitoes. Analysis of the oil by GC-MS showed it to be a rich source of five monoterpenes, thymol, *p*-cymeme, carvacrol, linalool and α-terpinene in order of amount. The five monoterpenes were individually assessed to determine their repellent activities to the mosquito on the mice. α-Terpinene was most potent with a protection

rate of 97% at a concentration of 0.05% topical treatment. Because of potential differences between mouse and human skin the repellent effect of α-terpinene and the other thyme oil components against *Cx. pipiens pallens* are examined further on human forearm skin, herein. Additionally, the potential of α-terpinene to irritate eye and skin using rabbits is also reported.

The skin sensitivity and eye irritation studies showed α-terpinene did not cause any acute histological contra indications.

However, α-terpinene treatments caused some irritation to the cornea represented by opacity. The degree and severity of cloudiness based on time of exposure are listed in Table 6. Cloudiness of the cornea was detected at 1 d post-treatment in both saline washed and α-terpinene treated rabbits. The group treated with a saline wash (W group) showed less cloudiness of the cornea than the group not provided a saline wash (NW group). The cloudiness of the cornea dissipated with time after treatment. Cloudiness disappeared in all rabbits after 3 days in the W group and 7 days in the NW group. The α-terpinene solution obviously could cause some eye irritation as represented by cloudiness of the cornea. However, this abnormality eventually was alleviated without special treatment and could easily be prevented by a saline wash.

Other abnormalities to the eye caused by α-terpinene included redness, chemosis and discharge of the conjuctiva. Redness of the conjuctiva was detected after 1 d in both saline washed (W) and non-washed (NW) rabbits. The degree of redness in the conjuctiva in the W group was slightly less than that of the NW group. The redness of the conjunctiva decreased after exposure and disappeared after 3 d in the W group and 7 d in the NW group, with exception of one rabbit that took 10 d. The application of α-terpinene to the eye could cause some redness to the conjunctiva. However, this redness disappeared over time without any permanent damage. Moreover, the redness could easily be prevented with saline washing shortly after any accidental exposure.

Chemosis and discharge of the conjunctiva were observed 1 d after treatment in all tested animals (Table 6 and Figure 1). Figure 1 indicates most discharge formation on the treated eyes first one to 3 days after treatment. The 2% α-terpinene solution produced severe irritation on the eyes in the NW group. The index of acute ocular irritation (I. I. O. I) was 18.0 and 52.7 for W and NW group, respectively, on the first day after treatment. It occurred severely irritation on the eyes if not properly washed. However, chemosis and discharge of the conjunctiva even-

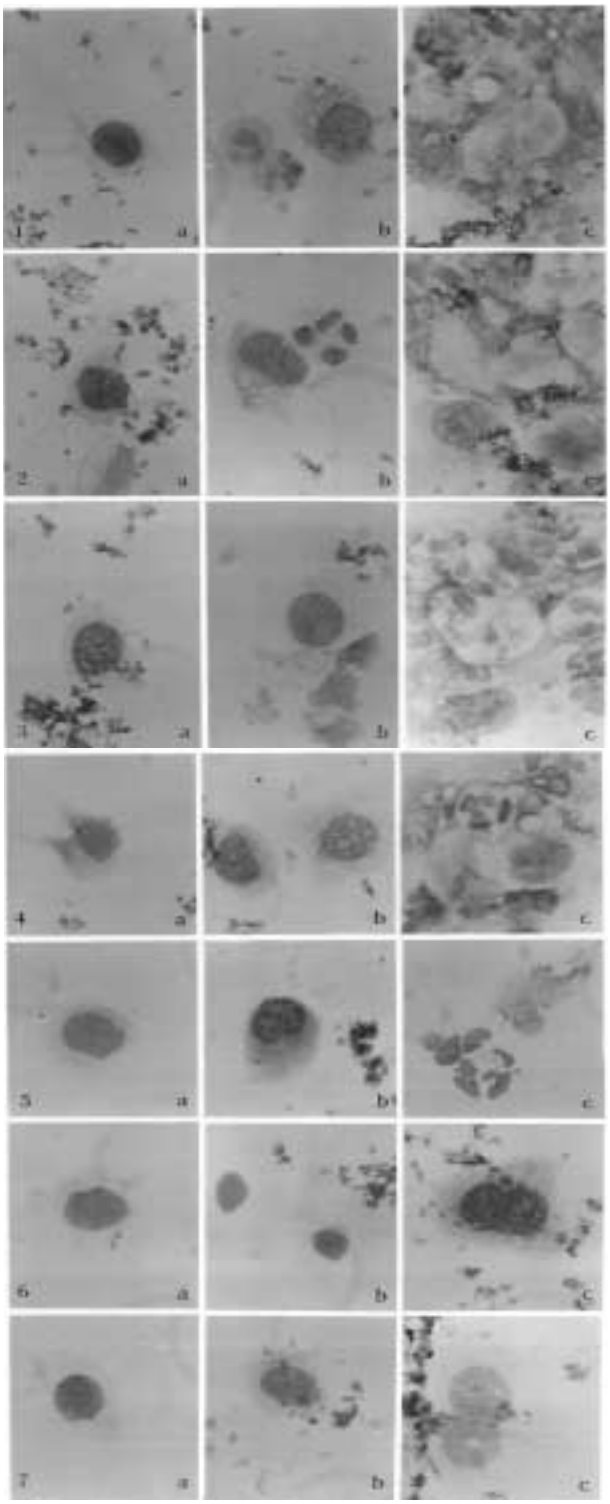

Figure 1. Conjuctival discharges of rabbit eyes stained with Giemsa: (1), 1 day; (2), 2 day; (3), 3 day; (4), 4 day; (5), 7 day; (6), 10 day; (7), 13 days after exposure to differing dose of α-terpinene on rabbit eye. (a) control (not treated); (b) exposure to α-terpinene but then washed with saline; (c) treated with α-terpinene without washing.

tually disappeared after exposure and could be prevented with saline washing. The main components of any ocular discharge in this study were fibrin, epithelial or epitheloid cells, lymphoid cells, erythrocytes and granulocytes. The relative amounts of and types of these discharge components in each group [non-treated group (NT), saline-washed group (W) and non-washed group (NW)] were listed in Tables 7 and 8. Fibrin was observed in all tested groups except for the NT group. The relative amount of fibrin observed in each of the groups (from lower to higher) was as follow: NT < W < NW. Fibrin in two discharge disappeared within 4 d in the W group and 7 d in the NW.

Epithelial or epitheloid cells were detected in the conjuctival discharge of all test rabbits. The increasing numbers in epithelial or epitheloid cells was observed to be in the following order: NT < W ≪ NW group. The occurrence of these cells in the conjuctival discharge was rare in the non-treated rabbits. However, large numbers of these cells were found in the NW group, suggesting some necrosis in the treated eyes. These abnormal conditions eventually declined post-treatment and saline washing significantly curtailed this necrotic effect.

Lymphoid cells were also detected in the W and NW groups. The appearance of such cells suggests some inflammation or hemorrhaging occurred in the eye as a result of exposure to α-terpinene. These cells disappeared from the conjuctival discharge at 3 d after treatment in the W group and 5 d in the NW group, with the exception of two rabbits.

Erythrocytes were also found in all rabbits in the NW group. Erythrocytes were observed in increasing numbers in the following order: NT < W ≪ NW groups. The occurrence of erythrocytes in ocular discharges indicates hemorrhaging occurred. Erythrocytes disappeared from the discharge 7 d after treatment in the NW group, except for one rabbit. Saline washing was effective in reducing the number of erythrocytes in the discharge.

Granulocytes were found in the conjuctival discharge of the W and NW groups, too. The types of granulocytes included numerous neutrophils and a smaller number of eosinophils. The appearance of neutrophils and eosinophils in the ocular discharge suggests supportive inflammation and allergic inflammation occurred, respectively, in the eyes. These cells disappeared, however, within 3 d after treatment in the W group and 7 d in the NW group.

Discussion

In a previous study, we found the essential oil of provided a 91% protection rate against biting by female *Cx. pipiens*, whereas the essential oils of eucalyptus, lavender and rosemary provided 70%, 65% and 77% protection, respectively, using hairless mice for bioassays[8]. All the essential oils tested exhibited significant repellent effects compared to controls. However, the essential oils of thyme were significantly more repellent than the other essential oils examined. Thus, the greater repellency in thyme resulted from compounds either absent or in low quantity in the other three essential oils. This study determined there were five major monoterpenes in the essential oil of thyme and among them α-terpinene was the most potent repellent against female *Cx. pipiens mosquitoes*. Based on the human forearm bioassay, α-terpinene provided 98% protection rate, whereas the commercial formulation of deet provided 89% protection rate. 1,8-Cineole present in the volatile oil of *Hemizonia fitchii* (Asteraceae) repelled mosquito feeding, moderately and ovipositioning strongly[8,9]. p-Methane-3,8-diols isolated from *Eucalyptus camaldulensis* were potent mosquito repellents and synthesized a new mosquito repellent, eucamalol, based on this structure[7]. Eucamalol was effective repellent (75%) up to 3 h after exposure to mosquito.

Until now, reports on skin irritation caused by α-terpinene or other monoterpenes against humans and animals were few. A report indicates high concentrations (above 10%) of monoterpenes, including α-terpinene, in ethanol evoked acute skin irritation in rabbits[10]. However, the irritation was not observed in lower concentrations of α-terpinene, below 5%. Also, they reported greater skin irritation of monoterpenes was in the following order: α-terpinene ≪ *t-p*-menthane = *d*-limonene < terpinolene ≪ azone. These results correspond to the findings in this present study, wherein no acute abnormalities were observed to intact or abraded skin after application of 2% α-terpinene. On the other hand, a report showed histopathological abnormalities in skin treated with a gel ointment containing 2% α-terpinene[11]. However, the observations are difficult to relate to this present study because of differences in the formulation and dosage type.

Reports of eye irritation in humans and animals caused by α-terpinene exposure are few. High concentrations (about 100%) of monoterpenes evoked eye irritation against humans and rape oilseed has been associated by rural populations to certain seasonal symptoms, such as coughing, headache and eye irritation[12,13]. It was determined that during its flowering season, the major volatile constituents emitted from rape oilseed were monoterpenes. However, α-terpinene was not one of the major volatiles. In this

present study, moderate and severe eye irritation occurred by a repellent solution containing 2% α-terpinene in the W and NW groups, respectively. These types and duration of eye abnormalities observed were transient spontaneously dissipated, and histological profiles returned to normal within 14 d. A repellent solution containing 2% α-terpinene should be considered as moderately to severely irritating to the eye. Alternatively, the potential eye irritation caused by a 2% solution of α-terpinene can be significantly curtailed by an immediate application of saline solution. Conclusively, α-terpinene in a 2% appears to be a potentially effective and safe mosquito repellent for humans.

Methods

Chemicals

N, *N*-diethyl-*m*-methylbenzamide, 2-cyano-1-methyl-3-{2-(5-methyl-imidazol-4-yl-methyl-thio) ethyl} guanidine (eudragit E100; a histamine antagonist), polyvinyl-pyrrolidone (povidone), polyethylene glycol 100 (PEG 100) were kindly provided by the Dong-Wha Co. (Seoul, Korea). α-Terpinene was purchased from Sigma (St. Louis, MO).

Animals

Male and female New Zealand white rabbits were obtained from Sejin Laboratory Co. (Seoul, South Korea) and were approximately 12-18 weeks of age on arrival at the laboratory. They were used for skin and eye irritation tests. All experimental animals were treated and experiments were conducted in accordance with the Korean Food and Drug Administration (KFDA) guidelines (98-116) in this study.

Primary Skin Irritation

Adult male (2.8-3.3 kg) and female (2.7-3.4 kg) New Zealand white rabbits were ascribed individual numbers marked in indelible ink on 1 ear. Rabbits were kept on a 12 h photoperiod and maintained at 61-70˚F and relative humidity of 40-70%, and provided water and food (Samyang Co. Ltd., Seoul, Korea). Animals were acclimated to environmental condition for 5 d before testing. Prior to skin-test fur was shaved from the dorsal trunk. Three males and three females were tested. A 1-inch square gauze patch was placed on the skin and secured with adhesive tape. A volume of 0.5 mL repellent solution (Table 1) was then applied to the patch. After applying the test compound polyethylene wrap was secured around the trunk of the rabbit. Rabbits were placed in a restraining cage for 4 h, after which wraps were removed.

Table 1. Formulation of test repellents topically applied to human forearms exposed to *Culex pipiens pallens* Coquilett.

Raw materials	Content portion (%)
Monoterpene (tested)	0.5 g (2.0%)
Eudragit E100	1.25 g (5.0%)
Povidone	0.5 g (2.0%)
PEG 400	0.05 g (0.2%)
Ethanol	22.7 g (90.8%)

Table 2. Scoring system used for measuring skin sensitization in the primary irritation study.

Effect	Value	Observation
Erythema	0	No erythema
	1	Very slight (barely perceptible)
	2	Well defined
	3	Moderate to severe
	4	Severe
Edema	0	None
	1	Very slight
	2	Slight
	3	Moderate (approximately 1 mm)
	4	Severe (>1 mm)

The local skin reactions were read and recorded at 24 h and 72 h using the scoring system shown in Table 2[14]. A skin biopsy was taken at 72 h after treatment for histological studies. Biopsies were taken after rabbits were sedated with intramuscular xylazine hydrochloride (5 mg/kg) and anesthetized with ketamine hydrochloride (35 mg/kg). The skin biopsies were fixed in 10% buffered formalin solution for several days, then underwent ethanol series dehydration and embedded in paraffin. Sections were stained with hematoxylin-eosin and examined under a light microscope.

Four different skin sensitivity cohorts were examined as follow; (1) non-treated at intact site (NTN group), (2) non-treated at abraded site (NTA group), (3) 2% α-terpinene solution at intact site (2TN group), and (4) 2% α-terpinene solution at abraded site (2TA group). Each test rabbit had 3 intact sites and 3 abraded sites.

Primary Eye Irritation

A separate group of adult male and female New Zealand white rabbits from those used in the skin sensitivity tests was used for eye irritation studies. Environmental conditions for rabbits were the same as those for rabbits used in the skin sensitivity study.

Repellent solution (0.1 mL) (Table 1) was dropped in the right eye of nine rabbits. Rabbits were divided

Table 3. Scales of weighted scores for grading the severity of ocular lesions.

1. Cornea	
A. Opacity - Degree of density (area which is most dense is taken for reading)	
Scattered or diffuse - details of iris clearly visible	1
Easily discernible translucent areas details of iris slightly obscured	2
Opalescent areas no details of iris visible, siae of pupilbarely discernible	3
Opaque. iris invisible	4
B. Area of cornea involved	
One quarter (or less) but not zero	1
Greater than one-quarter≈less than one-half	2
Greater than one-half≈less than three quarters	3
Greater than three quarters up to whole area	4
Score=A × B × 5	Total maximum=80
2. Iris	
A. Values	
Folds above normal, congestion, swelling, circumcorneal injection (anyone or all of these or combination of any thereof), iris still reacting to light	1
No reaction to light, hemorrhage, gross destruction (any one or all of these)	2
Score=A × 5	Total maximum=10
3. Conjunctive	
A. Redness (refers to palpebral conjuctiva only)	
Vessels definitely injected above normal	1
More diffuse deeper crimson red (individual vessels not easily discernible)	2
Diffused beefy red	3
B. Chemosis	
Any swelling above normal (inclused nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
C. Discharge	
Any amount different from normal (does not include small amount observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3
Score=(A+B+C) × 2	Total maximum=20

Table 4. Rating system used on sums of all scores obtained from ocular lesions.

Scores	Tentative ocular irritation rating
0 to 5 points	Non irritating
5 to 15 points	Mildly irritating
15 to 30 points	Moderately irritating
30 to 60 points	Severely irritating
60 to 80 points	Extremely irritating
80 to 110 points	Maximally irritating

Table 5. Grading system used to score amounts of each component in ocular discharges.

Grade	Remarks
0	Not detected
1	Rare
2	A few
3	Moderate
4	Numerous

Table 6. Results of eye reactions.

Observation	Group	Non-washed group (1-3)				Washed group (4-9)					
	Animal No.		1	2	3	4	5	6	7	8	9
Cornea	Degree of opacity (A)	1[a)]	1	1	2	3	3	4	2	3	1
		2	1	1	1	3	2	2	1	1	1
		3	0	0	0	2	2	1	1	1	1
		4	0	0	0	2	2	1	0	0	0
		7	0	0	0	1	1	0	0	0	0
		10	0	0	0	0	0	0	0	0	0
		13	0	0	0	0	0	0	0	0	0
	Diffuse are of opacity (B)	1	1	1	2	4	3	4	1	2	1
		2	1	1	1	3	2	3	1	2	1
		3	0	0	0	3	1	1	1	1	1
		4	0	0	0	1	1	1	0	0	0
		7	0	0	0	1	1	0	0	0	0
		10	0	0	0	0	0	0	0	0	0
		13	0	0	0	0	0	0	0	0	0
	Iris (C)	No change				No change					
Conjuctiva	Redness (D)	1	1	1	1	3	3	2	2	2	2
		2	1	1	1	3	3	2	2	1	2
		3	0	0	0	2	2	1	1	1	1
		4	0	0	0	1	1	0	0	0	0
		7	0	0	0	1	0	0	0	0	0
		10	0	0	0	0	0	0	0	0	0
		13	0	0	0	0	0	0	0	0	0
	Chemosis (E)	1	2	1	1	2	4	2	2	3	1
		2	1	0	0	1	2	2	1	2	1
		3	0	0	0	1	1	1	0	0	1
		4	0	0	0	1	0	0	0	0	0
		7	0	0	0	0	0	0	0	0	0
		10	0	0	0	0	0	0	0	0	0
		13	0	0	0	0	0	0	0	0	0
	Discharge (F)	1	1	2	2	3	3	3	2	2	2
		2	1	1	1	3	3	3	2	2	2
		3	0	0	0	3	3	2	1	1	1
		4	0	0	0	2	1	1	1	1	1
		7	0	0	0	1	0	0	0	0	0
		10	0	0	0	0	0	0	0	0	0
		13	0	0	0	0	0	0	0	0	0
	I.O.I Score[b)]	1	13	13	28	76	65	94	22	44	15
		2	11	9	9	61	36	44	15	20	15
		3	0	0	0	42	22	13	9	9	11
		4	0	0	0	18	14	7	2	2	2
		7	0	0	0	9	5	0	0	0	0
		10	0	0	0	0	0	0	0	0	0
		13	0	0	0	0	0	0	0	0	0
	M.I.O.I Score[c)]	1		18.0					52.7		
		2		9.7					31.8		
		3		0					17.7		
		4		0					7.5		
		7		0					2.3		
		10		0					0		
		13		0					0		
	I.I.O.I Score[d)]	1		18.0 (Moderately irritating)					52.7 (Severely irritating)		

[a)]Observation Time (days after treatment).

[b)]The individual index of ocular irritation (I.O.I. score) was calculated by $(A \times B \times 5)+(C \times 5)+2(D+E+F)$.

[c)]M.I.O.I. scores indicate mean index of ocular irritation. [d)]I.I.O.I scores indicate the index of acute ocular irritation.

Grade of scores were listed in Table 4. Note that the scores in the non-treated control were all zero.

Table 7. Components and amounts in ocular discharges of eyes in rabbits cohort treated with 2% α-terpinene than washed with saline.

Days	Fibrin	Epithelial cells	Lymphoid cells	Erythrocytes	Granulocytes
1	1.33±0.58[a]	2.00±1.00	2.33±0.58	0.00±0.00	2.67±0.58
2	1.33±0.58	1.67±0.58	0.67±0.53	0.00±0.00	1.33±0.58
3	1.00±0.00	1.67±0.53	0.00±0.00	0.00±0.00	0.33±0.58
4	0.00±0.00	1.33±0.58	0.00±0.00	0.00±0.00	0.00±0.00
7	0.00±0.00	1.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
13	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Explanation of scores was listed in Table 5. Note that the scores in the non-treated control were all zero except for epithelial cells, which were 1, 2, 1, 1, 1, 1 and 1 at 1, 2, 3, 4, 7, 10 and 13 days after respectively. [a)]Mean±S. D. (n=3).

Table 8. Components of ocular discharges in the eye of Non-Washed group after 2% α-terpinene solution dropping.

Days	Fibrin	Epithelial cells	Lymphoid cells	Erythrocytes	Granulocytes
1	2.87±1.47[a]	2.67±0.82	2.17±0.75	1.67±0.82	3.33±1.21
2	2.50±0.55	2.83±0.75	1.67±0.52	1.50±0.55	2.17±1.33
3	1.67±0.82	2.33±0.82	0.83±0.41	0.83±0.75	1.83±1.17
4	1.17±0.41	1.67±0.82	0.33±0.52	0.50±0.55	1.33±1.03
7	0.33±0.52	1.33±0.52	0.00±0.00	0.17±0.41	0.17±0.41
10	0.00±0.00	1.33±0.52	0.00±0.00	0.00±0.00	0.00±0.00
13	0.00±0.00	1.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Explanation of scores was listed in Table 5. Note that the scores in the non-treated control were all zero except for epithelial cells, which were 1, 2, 1, 1, 1, 1 and 1 at 1, 2, 3, 4, 7, 10 and 13 days after respectively. [a)]Mean±S. D. (n=6).

into two groups. One group of three rabbits was washed with 20 mL of warmed distilled saline (W group). A second group of six rabbits did not have their eyes flushed with saline (NW group). The eyes or both groups were subsequently examined for indication of ocular and periocular trauma and/or inflammation at 1, 2, 3, 7, 10 and 13 days after treatment. Effects were scored according to the system shown in Tables 3 and 4.

Ocular discharge was collected using pasteurized swabs at 1, 2, 3, 4, 7, 10 and 13 days after treatment. Ocular discharges were smeared on to a glass slide and fixed for 20-30 min using methanol. After fixing, slides were stained with Giemsa solution for 20-25 min. Stained slides were observed under a light microscope. Cellular components of ocular discharges were determined and numbers of each component were scores. The range in amounts of each component in ocular discharges is listed in Table 5.

Eye tissues were sampled by biopsy at 14 days after treatment. Rabbits were sedated with intramuscular xylazine hydrochloride (5 mg/kg) and anesthetized with ketamine hydrochloride (35 mg/kg). Biopsies of the eyes were sampled and fixed in Bouin solution for several days. The eyes then underwent ethanol series dehydration and the tissue was embedded in paraffin. Sections were stained with hematoxylin-eosin for observation of the cornea, iris and retina.

After treatment, I.I.O.I (the individual index of ocular irritation), M.I.O.I. (Mean index of ocular irritation) and I.A.O.I. (the index of acute ocular irritation) were calculated following a guideline of Korea Institute of Toxicology (Daejeon, Korea).

Statistical Analyses

Data collected during evaluation of biting-rate and duration-of-protection tests were subjected to Scheffe's test ($P=0.05$)[15].

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