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Research paper

Subconjunctivally injected, liposome-encapsulated streptokinase enhances the absorption rate of subconjunctival hemorrhages in rabbits

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ABSTRACT

Liposome-encapsulated streptokinase (SK) was prepared with distearoylphosphatidylethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG_{2000}). In vitro release assay demonstrated over 81% of SK was released from liposomes at 48 h, and the effect of its subconjunctival injection on the absorption rate of induced subconjunctival hemorrhage (SH) in rabbits was evaluated. After 8 h of SH induction, eyes were randomly assigned to one of four subconjunctival injection groups (10 eyes each): group A: the free form of SK (1000 IU/mL); group B: liposome-encapsulated SK (1000 IU/mL); group C: 0.1 mL of liposomes; and group D: no injection. SHs were photographed at 8, 24, 48, 72, and 120 h after SH induction and their sizes were compared. Size decrease of the SH was faster in groups A and B than in groups C and D. Group B displayed significantly different absorption rates than group A at 24 and 48 h and with groups C and D at 24, 48, and 72 h, with the shortest mean elapsed time among all groups. The ocular absorption of SK was lower after the injection of the liposome-encapsulated SK than the free form. These results demonstrated that subconjunctival injection of liposome-encapsulated SK enhances the rate of SH absorption, especially in the early phases.

1. Introduction

Streptokinase (SK, m.w. 47 kDa) is one of the most widely used and least expensive fibrinolytic agents used for treating thromboembolic conditions [1], which counteracts with fibrin thrombus formation by converting proenzyme plasminogen into plasmin [2]. SK has a half-life of about 20–30 min after intravenous injection [3,4] due to its rapid clearance from circulation by antibodies and the reticuloendothelial system [4]. Liposomes have been used to improve the systemic delivery of drugs with short biological half-lives by protecting them from degradation in vivo [4], and SK-bearing liposome composed of cholesterol and distearoylphosphatidylethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG_{2000}) was shown to have 16-fold increased half-life of 5.4 h in rat plasma [4].

Subconjunctival hemorrhage (SH) is a common ophthalmic condition resulting in a troublesome cosmetic disfigurement. However, there has not been an approved therapeutic measure to enhance its absorption in ophthalmologic practice. There has been only one animal study reported from our laboratory with low-molecular-weight heparin (LMWH) [5], while few therapeutic trials have been attempted to enhance its absorption in the past [6].

Subconjunctival liposomes were reported to be associated with the sustained release and retentive effects of encapsulated drugs at the site of injection in animal models [7,8]: single subconjunctival injection of liposome-encapsulated antibiotics indeed demonstrated better effect than a higher dose of liposome-free antibiotics in a rabbit *Pseudomonas* keratitis model [7]. Subconjunctival injection might be the best way of local treatment for SH to increase the local concentration of the drug in the desired location while decreasing the systemic absorption and/or side effect of the drug, and sustained release of SK from liposomes may be helpful in facilitating the absorption of SH. Therefore, we planned to investigate the effect of subconjunctival injection of liposome-encapsulated SK on the absorption rate of induced SH in rabbits, and monitored the possible side effects of SK including systemic reaction and drug absorption into plasma and intraocular tissues at different time points.

2. Materials and methods

2.1. Materials and animals

Streptokinase (250,000 IU), cholesterol-3-sulfate (CHS), D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride (S-2251), soybean trypsin

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inhibitor (SBTI) and thrombin were purchased from Sigma (St. Louis, MO, USA). Distearolyphosphatidylcholine (DSPC), cholesterol (CH) and distearoylphosphatidylethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG_{2000}) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Human plasminogen (120 U) was purchased from Merck (Darmstadt, Germany). Fifty New Zealand white rabbits, weighing 2.0 to 3.0 kg, were used as animal models. All experimental methods and animal care procedures were reviewed and approved by the institutional committee for animal studies at Kim's Eye Hospital.

2.2. Preparation of liposome-encapsulated SK

Liposome-encapsulated SK was prepared by the freeze-thawing method [9]. Briefly, lipid mixture (90 μmol total lipid) composed of DSPC, CH, CHS and DSPE-PEG_{2000} in a molar ratio of 1.85:0.85:0.15:0.15 was dissolved in the mixture of organic solvents ($CHCl_3$:MeOH = 2:1, v/v). The organic phase was removed using a rotary evaporator under a reduced pressure (360 mmHg) and liposomes were prepared by hydrating the lipid film with 2 mL of Tris–Cl buffer (pH 7.4, 0.05 M Tris, 0.01 M NaCl). The liposomes were subsequently frozen in liquid nitrogen, and thawed at room temperature with 100 μL of SK (100,000 IU/mL). After five freeze–thawing cycles, and the resulting liposomes were extruded 10 times through a 400 nm polycarbonate membrane (Whatman, Clifton, NJ, USA) under nitrogen pressure (300 kPa). The un-encapsulated SK was then separated by removing the supernatant after ultracentrifugation in a rotor (TLA 100.3; Beckman) at 150,000*g* for 1 h. Liposome-encapsulated SK was finally formed by resuspending liposome pellet, and the suspension was subsequently diluted in Tris–HCl buffer to make the final concentration of 1000 IU/mL and was stored at 4 °C.

2.3. Physical properties of SK-bearing liposomes

The mean particle size and polydispersity index (PI) of SK-encapsulating liposomes were determined by dynamic light scattering method using electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan) at a fixed angle of 90° and at room temperature. Prior to measurement, liposomes were diluted with filtered deionized water. The system was used in the auto-measuring mode. The laser diffraction particle size analysis data were evaluated using volume distribution to detect even a few large particles. The PI is a measure of the distribution of particulate population. Small PI means the narrow distribution of liposomes.

The electrophoretic mobility of liposomes was determined using electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan). The electrophoretic mobility was measured after the dilution of samples with filtered deionized water. The measured electrophoretic mobility data were converted into zeta potential using Helmholtz–Smoluchowski equation. The processing was done by the software included within the system.

Encapsulation efficiency of SK in liposomes was determined by measuring the SK activity after disrupting the liposomes with 0.1% Triton X-100. The SK activity was determined according to the previously reported method [10] with a slight modification. The determination of SK activity is based on the rate of amidolysis of the chromogenic substrate, S-2251, by the SK-plasminogen activator complex. The assay procedure was as follows: 470 μl of Tris–HCl buffer (pH 7.4, 0.05 M Tris, 0.01 M NaCl), 80 μl of SBTI (0.2 mg/mL), 100 μl of plasminogen (10 U/mL) and 50 μl of liposome solution pretreated with 0.1% Triton X-100 were mixed in a silanized glass tube at 37 °C. SK standard of 100 μl was added and the timer started. The concentration range of SK standard was between 20 and 1000 IU/mL. At 4 min after the addition of standard SK, the mixture was transferred to a 1 mL cuvette in a thermostated cuvette holder in a spectrophotometer and at 5 min, 200 μl of S-2251 (3.3 mmol/L) was added. Then the mixture was shaken and the rate of amidolysis was recorded for 5 min. Maximum optical density change (ΔOD) at 405 nm was determined. The resulting maximal ΔOD was converted to SK activity in IU/mL according to the standard curve equation. Physical properties of liposome formulations are summarized in Table 1.

2.4. Evaluation of in vitro release of SK from liposomes

The SK-bearing liposomes were placed in a closed vial and immersed in shaking water bath at 37 °C. At 12, 24, 48, 72, 96, and 120 h after the preparation of SK-bearing liposomes, in vitro release rate of SK from liposomes was determined by separating liposomes from the suspension medium by ultracentrifugation at 150,000*g* for 1 h. The supernatant was removed and replaced with the same quantity of sterile saline for the subsequent analysis. SK content in the supernatant was determined by an assay for SK activity as described above [10]. The cumulative release fraction was defined as follows: $(C/T) \times 100$ (%), where C is the cumulative amount of SK detected only in the supernatant, and T is the total amount of SK in liposomes.

2.5. Induction of SHs and evaluation of the SH absorption rates

Animal experiments were conducted using the previously described rabbit SH model [5] with a slight modification. General anesthesia was achieved by intramuscular injection of 10–15 mg/kg of Zoletil® (5–7.5 mg/kg of tiletamine and 5–7.5 mg/kg of zolazepam: Virbac Laboratories, France) and 5–10 mg/kg of xylazine, and proparacaine hydrochloride was used for topical anesthesia. Autologous blood was sampled from the auricular veins and was injected subconjunctivally into both eyes of all rabbits (0.05 mL, 1–2 mm from the superior limbus).

Eight hours after the SH induction, the material to be injected for each eye of the rabbits was assigned by block randomization: group A (10 eyes): 0.1 mL of the free form of SK (1000 IU/mL); group B (10 eyes): 0.1 mL of liposome-encapsulated SK (1000 IU/mL); group C (10 eyes): 0.1 mL of liposomes; and group D (10 eyes): no injection. A preliminary experiment demonstrated no significant effects on the SH absorption rate in the fellow untreated eyes after the treatment of one eye with one of the regimens, thus allowing simultaneous use of both eyes. The participants, who performed block randomization, subconjunctival injection and measurement of the SH sizes, were blind to the types of the injected materials.

The image of SH was photographed with a digital camera (PowerShot® A85: Cannon, Tokyo, Japan) at 8, 24, 48, 72, 96, and 120 h after the hemorrhage induction with a translucent bar marked with 1-mm graduations as a reference for sizing. The sizes of digitalized SH images were measured using an image analyzer program (Image Pro-Plus, ver. 6.0; Media Cybernetics, Silver Spring,

Table 1
Physical properties and encapsulation efficiency of liposome formulations

	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
Liposome	236.1 ± 9.3	0.182 ± 0.021	−22.62 ± 4.34	–
Liposome-SK[a]	245.9 ± 13.3	0.190 ± 0.013	−26.99 ± 5.21	20.87 ± 2.72

The results are expressed as the mean ± SD (n = 5).
[a] Liposome-SK, liposome-encapsulated SK, SK-bearing liposomes.

MD), and the SH size at 8 h after the induction was defined as the basal level of each case.

Two parameters were used for the evaluation of SH absorption rates. One was the amount of decrease in the size of SHs from the baseline size (i.e., the size of hemorrhage at 8 h after the induction), which was compared between groups at 24, 48, 72, and 120 h after the SH induction. The other parameter used was the mean elapsed time required for the complete absorption of SH. The actual time when SH was no longer observed was recorded, and the elapsed time required for the complete SH absorption was calculated by abstracting the time of SH induction. All the animals were euthanized with pentobarbital sodium overdose after SHs were completely absorbed.

2.6. Evaluation of the ocular and systemic absorption and intraocular and systemic side effects of SK

Eighteen rabbits were used to evaluate the safety of the drug injection. Eight hours after the SH induction in one eye of each rabbit (18 eyes), subconjunctival injection of the free form of SK (0.1 mL × 1000 IU/mL: 9 rabbits) or liposome-encapsulated SK (0.1 mL × 1000 IU/mL: 9 rabbits) was randomly performed.

Systemic side effects were checked for its occurrence every day according to the criteria for anaphylactic reactions [11], which include restlessness, piloerection, sneezing, and others until each animal was sacrificed. Slit lamp and fundus examinations were performed after pupil dilation using mydriatic eye drops before and at 24, 72, and 240 h after the SH induction. The development of ocular side effects including conjunctival injection, corneal or lens opacity, anterior chamber hemorrhage or inflammation, and vitreous hemorrhage or inflammation was monitored.

Three animals were randomly chosen from each group at 24, 72, and 240 h after the SH induction and were sacrificed for an assay of SK activity in the plasma and ocular tissues. Tenth day time point was chosen because the neutralizing antibody titers and activities against SK have been reported to rise much higher in 7–10 days after SK treatment for acute myocardial infarction compared to 3–4 days after the same treatment [12]. After general anesthesia, 10 mL of venous blood was sampled and collected in citrated containers. Plasma was separated after centrifugation and was stored in −70 °C freezer until being analyzed.

The rabbits were then euthanized and the eyes were enucleated. To evaluate the ocular absorption of SK in the treated and untreated fellow eyes, the SK activity in ocular tissues was measured. Aqueous humor, vitreous, and pieces of conjunctiva and sclera adjacent to the injection site were harvested and kept in tubes at −70 °C for the subsequent analysis. The tissues obtained from vitreous, sclera and conjunctiva were chopped into small pieces and pooled separately in a 1.5-mL Eppendorf tube containing 0.5 mL of pH 7.4 phosphate-buffered saline. The tubes were incubated for 30 min, vortexed for 1 h, and were centrifuged for 30 min at 10,000*g* to separate SK from the tissues. The amount of SK in the supernatant separated from each tissue and in aqueous was determined with an assay for SK activity.

The concentration of SK in plasma was determined by an assay for SK activity, and SK resistance by neutralizing antibody was evaluated by the previously reported neutralization assay [13] with a slight modification. Briefly, SK neutralization titers were measured using samples of frozen plasma as follows. SK was diluted in physiological saline to give a range of concentrations from 1 to 1000 IU/mL. Diluted SK of 10 μL was mixed with 100 μL frozen plasma sample in a 12 × 75 (mm) tube, and 5 μL of bovine thrombin (100 U/mL) was added. The formation of plasma clot was confirmed, and the tubes were immediately placed in a water bath at 37 °C for 10 min. The lowest concentration of SK that caused the clot to lyse within 10 min was recorded, and the final SK titer in the tube was calculated from this concentration as: the dilution of SK in tube/10, where 10 is the dilution factor.

2.7. Statistical analysis

All continuous variables were expressed as the mean ± SD. The difference in the amount of SH size decreases between the groups was evaluated with repeated-measures ANOVA. The baseline sizes and mean elapsed times for the complete absorption of SHs were compared with one-way ANOVA. Post hoc pair-wise comparisons were performed among the four groups. Systemic and ocular absorption of SK were compared by Mann–Whitney *U*-test. All probability values were two-tailed and the level of significance was considered to be $P < 0.05$. Statistical analyses were performed using the SPSS software (SPSS 15.0; SPSS, Chicago, IL, USA).

3. Results

3.1. In vitro release of SK from liposomes

In a previous study, SK incorporated in liposome showed prolonged amidolytic activity in rat plasma compared with that of SK alone [4]. In this experiment, we evaluated in vitro release of SK from liposome since the characteristics of subconjunctival space would be different from those of intravascular space. The SK-bearing liposome was shown to release SK in a two-phasic manner: a high release step followed by a low release phase. The release of SK from liposomes reached almost the peak level of 81.54 ± 2.97% at 48 h and did not increase much after the time point. The cumulative release fraction of SK from liposomes by time is presented in Fig. 1.

3.2. Size decrease rate of SHs

The size decreases of the SHs are presented in Table 2. The baseline sizes of SHs at 8 h after the hemorrhage induction (mean ± SD) were 45.75 ± 3.39, 44.29 ± 2.57, 43.87 ± 2.00, and 45.67 ± 3.66 mm^2 in groups A, B, C and D, respectively, which showed no statistical differences among all groups ($P = 0.389$ by ANOVA). Yet, decreases in SH sizes in the four groups along all time points were significantly different ($F = 35.07$ for grouping factor, $P < 0.001$ by repeated-measures ANOVA). Size decrease of the SHs was faster in groups A and B, and the major differences among groups were mostly observed in the earlier phase of the study, i.e., until 72 h.

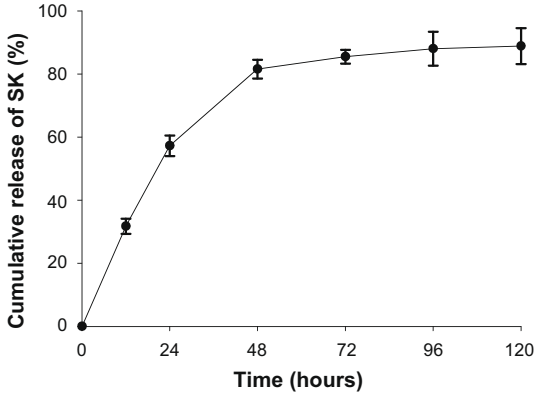

Fig. 1. The cumulative rate of in vitro release of SK from liposome formulation at 12, 24, 48, 72, 96, and 120 h after its preparation. Data are expressed as the mean ± SD. The results suggest that most of SK is released from the liposomes within 48 h after its preparation ($n = 3$).

Table 2
Size decrease of subconjunctival hemorrhages

Time (h)	Group A	Group B	Group C	Group D
24	13.13 ± 4.18	28.57 ± 5.66	7.80 ± 2.05	3.78 ± 2.05
48	30.09 ± 5.71	36.19 ± 6.21	14.43 ± 2.97	8.41 ± 3.07
72	39.59 ± 2.93	40.63 ± 4.63	31.19 ± 6.89	32.83 ± 7.02
96	44.85 ± 3.32	43.21 ± 3.29	41.31 ± 3.09	40.80 ± 3.69
120	45.75 ± 3.39	44.29 ± 2.57	43.45 ± 2.03	45.54 ± 3.76

Data are expressed as the mean reduction from baseline size (mm^2) ± SD.

Post hoc pair-wise multiple comparisons adjusted by the Bonferroni method are presented in Table 3. Significant differences in the SH size decreases among groups were limited to the earlier phase of the study, i.e., until 48 to 72 h after the hemorrhage induction. Group B revealed significantly faster SH size decrease than all the other groups at 24 and 48 h after the induction ($P < 0.001$ for groups A, C, and D at 24 h; $P = 0.040$ for group A and $P < 0.001$ for groups C and D at 48 h, respectively), and its size reduction rate was faster than groups C and D at 72 h after the hemorrhage induction ($P = 0.004$ for group C, $P = 0.023$ for group D). The SH size decrease in group A was significantly faster than that in groups C and D at 24 and 48 h after the induction ($P = 0.021$ and $P < 0.001$ for groups C and D at 24 h, $P < 0.001$ for groups C and D at 48 h, respectively), and at 72 h after the induction, the difference only occurred between groups A and C ($P = 0.012$). The differences between groups A and B were significant at 24 and 48 h after SH induction ($P < 0.001$ and 0.040 for 24 and 48 h, respectively), and such differences were not observed from 72 h time point.

3.3. Elapsed time for complete SH resorption

The mean elapsed times (mean ± SD) for the complete absorption of the SH are 109.60 ± 17.33, 92.70 ± 19.29, 117.60 ± 21.01, and 117.60 ± 20.87 h for groups A, B, C, and D, respectively, and were significantly different between the groups ($F = 3.861$, $P = 0.017$). The mean times were shorter in groups A and B than in groups C and D. In the post hoc pair-wise comparisons, only group B exhibited significant difference from groups C and D (Fig. 2).

3.4. Evaluation of ocular and systemic absorption and intraocular and systemic side effects of SK

No clinically detectable systemic and ocular side effect was developed in any animal throughout the study after the injection of liposome-encapsulated or free SK. Systemic absorption after subconjunctival injection of free or liposome-encapsulated SK was not detected; SK activity in plasma was not detected in both groups at 24, 72, and 240 h after the SH induction and SK neutralizing titer was less than 0.1 SK IU/mL in both groups before and at 24, 72, and 240 h after the SH induction.

Table 3
Post hoc pair-wise multiple comparison of the size decrease of subconjunctival hemorrhages

Multiple comparisons among groups		Time (h)				
		24	48	72	96	120
A	B	0.000[a]	0.040[a]	1.000	1.000	1.000
	C	0.021[a]	0.000[a]	0.012[a]	0.142	0.576
	D	0.000[a]	0.000[a]	0.065	0.062	1.000
B	C	0.000[a]	0.000[a]	0.004[a]	1.000	1.000
	D	0.000[a]	0.000[a]	0.023[a]	0.699	1.000
C	D	0.142	0.044[a]	1.000	1.000	0.781

Data are probabilities obtained with one-way ANOVA at each time.
[a] $P < 0.05$.

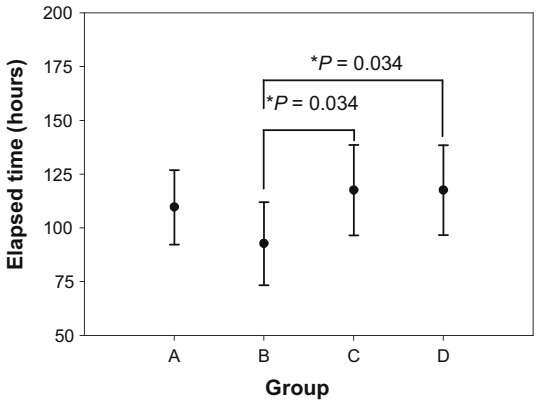

Fig. 2. Post hoc groupings after comparison of the mean elapsed time for the complete resorption of SHs among the four study groups. The mean elapsed times were significantly different between the groups ($F = 3.861$, $P = 0.017$). The mean times were shorter in groups A and B than in groups C and D. However, in the post hoc pair-wise comparisons adjusted by the Bonferroni method, only group B exhibited significant difference from groups C and D. Data are expressed as the mean ± SD. The groups with the $^{*}P$-values are significantly different from each other.

SK activities in various ocular tissues are presented in Fig. 3. Since we did not use any detergent to extract SK from the liposomes, the SK activity measured should be the activities outside the liposomes only. SK activities in sclera and conjunctival tissue seemed to be higher in free SK group than in SK-liposome group at 24 h after the SH induction; however, the difference was not significant. There was little SK activity in sclera and conjunctiva at 72 h after the induction only in SK-liposome group, and the SK activity was negligible at the other time points (Fig. 3A). The SK activities in the aqueous humor and vitreous of eyes treated with free SK were higher than those treated with SK-liposome at 24 and 72 h after SH induction. However, the difference reached statistical significance only in aqueous humor at 24 h after SH induction ($P = 0.046$, Fig. 3B) and marginal significance in vitreous at 24 and 72 h after the SH induction ($P = 0.05$, Fig. 3C). There was no detectable SK activity in the fellow untreated eye tissues after the injection of either drug throughout the study.

4. Discussion

In this study we have demonstrated that subconjunctival injection of liposome-encapsulated SK enhances the rate of SH absorption in the early phases, with minimal intraocular and no systemic absorption in rabbits. Free form of SK injection also exhibited similar effects; however, it demonstrated longer elapsed time for complete SH resorption and more intraocular absorption. This would be the first report of SK used as liposome-encapsulated forms for facilitating the absorption of SHs in the eye.

The SH absorption rate in liposome-encapsulated SK injection group was faster than that in free SK group, especially in the early phases (24–48 h after SH induction, which is 16–40 h after the drug injection), and then the difference between the two groups became non-significant. This early effect of the drug coincides well with the in vitro release data which demonstrated over 81% of SK was released from liposomes at 48 h, and it is appropriate for SH where spontaneous absorption occurs without any intervention; the time for complete SH absorption was also about 5 days in liposome injection and no injection groups. It seems that both forms of the drug exerted their effect mostly in the very early phase of the

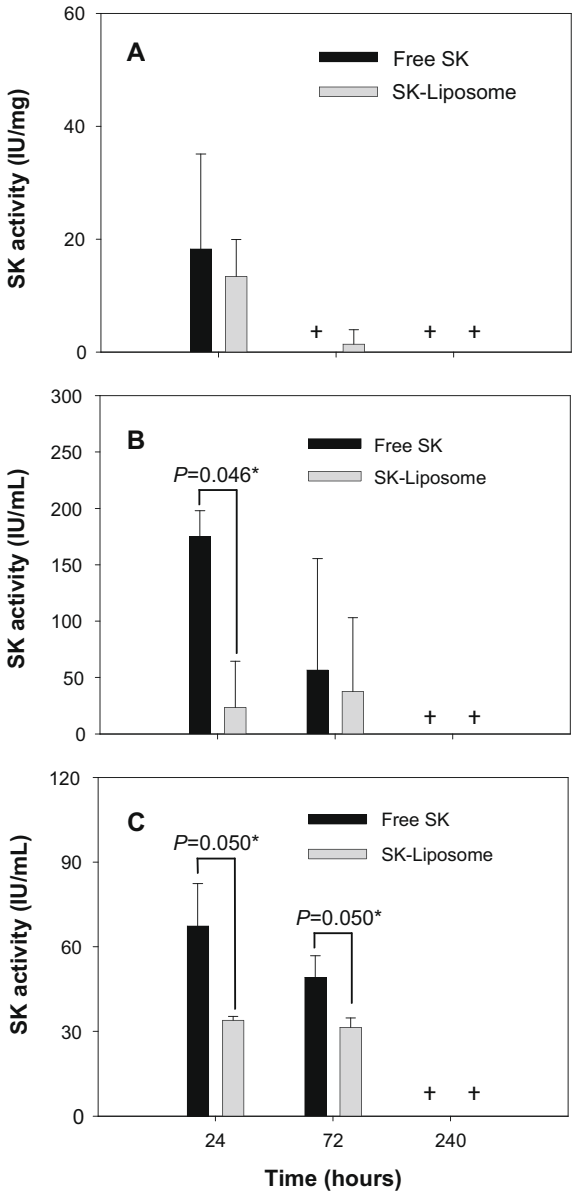

Fig. 3. Comparison of the SK activity in sclera-conjunctiva (A), aqueous humor (B), and vitreous (C) between eyes treated with free form of SK (SK) and liposome-encapsulated SK (SK-liposome) at 24, 72 and 240 h after SH induction. Data are expressed as the mean ± SD. †Values were not detected. P-values are calculated by Mann–Whitney *U*-test. $^{*}P \leqslant 0.05$.

study, and liposome-encapsulated SK have shown longer effect than free SK possibly due to its sustained release from liposomes [4]. More sustained SK activity in the subconjunctival space of SK-liposome group is expected since there was a little of SK activity in sclera and conjunctiva in the group at 72 h time point while there was none at all in the free SK group (Fig. 3A). The SK-liposome injection group could have sustained and lower SK activity than free SK injection group, which could be better for fibrinolysis because of the double-step plasminogen activation mechanism of SK [14]; SK first forms an equimolar complex with plasminogen, then this activator complex converts free plasminogen into plasmin. High dose of SK might leave only a small amount of free plasminogen available for the conversion into plasmin [14] after forming complexes with most of the inactive plasminogen in the blood clot.

Subconjunctivally administered drug can be absorbed into the eye via corneal penetration, systemic absorption, and trans-scleral absorption [15]. Different drugs demonstrated different ocular distributions after subconjunctival injection. Also liposomal encapsulation of the drug or the status of the eye would change drug distribution after injection [16]. Gentamicin exhibited increased level of the drug in cornea after subconjunctival injection of liposome-encapsulated forms [8], whereas tissue plasminogen activator (tPA, 70 kDa) demonstrated higher vitreous levels than aqueous levels after subconjunctival injection in gas vitrectomized rabbit eyes [15]. In this study, SK activity was displayed in various ocular tissues including sclera-conjunctiva, vitreous and aqueous humor, however not in the plasma. SK activities in the vitreous and aqueous were higher in SK group than in the SK-liposome group at 1 day and 3 days after subconjunctival injection. Sclera is permeable to molecules as large as 70 kDa [17,18], hence, free form of SK (47 kDa) would easily diffuse through sclera into vitreous. Free SK would also diffuse across cornea into aqueous humor more easily than liposome-encapsulated SK, and there would be less amount of free SK in the liposome-encapsulated SK group than in SK group, therefore more amount of SK in aqueous humor and vitreous can be detected in SK group than in SK-liposome group. Plasma SK activity was not detectable in both groups at any time after the injection, and part of the reason could have been the very small amount of the drug we administered.

SK is an agent with potentially systemic action and is known to have antigenicity to human causing allergic reactions [13,19,20]. Allergic reactions and formation of the neutralizing antibodies may not depend on the dosage of drug used, thus we investigated the systemic absorptions and side effects of the drug. Since mild allergic reactions reported in humans such as urticaria, itching, flushing, nausea, headache, and muscular and skeletal pain [19] are not practical to monitor in rabbits, hence, signs of anaphylactic reaction in animals [11] were monitored instead, which were not shown throughout the study. Neutralizing antibodies to SK are known to develop in most patients treated with SK for acute myocardial infarction [12,13], and SK neutralization titers were reported to increase even after the application of topical SK to wounds [21]. SK neutralizing antibodies are of clinical concern because they can lead to treatment failure with SK and can possibly be related to allergic reactions including potentially fatal anaphylaxis. However, the studies about the relationships between the antibodies and the undesirable effects have exhibited inconsistent results [22,23], and the SK neutralization activities below the detection level (0.1 SK IU/mL in this study) would not cast a serious clinical concern in terms of SK activity and safety of this regimen when low titers of anti-SK antibody are already widespread in the human population [24].

Few limitations to this experiment are the followings. First of all, as we measured the area of SH without considering height and curvature of the lesions, the measured size may not have reflected the actual amount of the hemorrhage. To minimize this error, we took the baseline pictures of SH at 8 h after the induction when the convex SH lesions were flattened and tried to take the picture right above the center of the lesion at right angle with a reference ruler to control the magnification factor. Secondly, the elapsed time required for the complete absorption of SH was calculated using the actual time recorded when SH was no longer observed, not the exact moment when all SH was completely disappeared. The time when SH was no longer observed must have come after the exact moment of SH disappearance, and the resultant elapsed time would be longer than the real elapsed time. Nonetheless, we tried to reduce errors in time measurement by examining the rabbits several times a day especially when the SH is almost absorbed, and significant differences in time between the groups could be elucidated. Thirdly, the actual concentration of SK itself was not measured; instead, we measured the activity of SK by detecting the amidolysis rate by the SK-plasminogen activator

complex [10]. Nonetheless, the side effects of SK would probably be mediated by free SK, not by SK within liposomes, and we believed that measuring the activity of SK was an appropriate approximation in evaluating its side effects. Fourthly, this study focused on verifying the safety of treatment and did not evaluate SK activities in tissues in very early phase after drug injection. Lastly, the amount of SH in 0.05 mL of blood might not stand for the sufficient volume of clinically significant SH in humans; therefore, the dose of liposome-encapsulated SK in this study (1000 IU/mL) would need to be adjusted depending on the amount of blood involved with SH in clinical settings.

In conclusion, the subconjunctival injection of liposome-encapsulated SK enhances the rate of SH absorption in the early phases, with minimal intraocular and no systemic absorption causing no side effect in rabbits. Given the non-detectable levels of plasma SK neutralizing activity and minimal SK activity in ocular tissues, a small amount of this drug could be attempted to be used to facilitate the absorption of SH in humans with a minimal risk of SK resistance or allergy.

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