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Preparation and evaluation of sinomenine hydrochloride *in situ* gel for uveitis treatment

Jike Song [a], Hongsheng Bi [b,*], Xiaofeng Xie [b], Junguo Guo [c], Xingrong Wang [b], Damei Liu [a]

[a] Shandong University of Traditional Chinese Medicine, 16369#, Jingshi Road, Jinan 250014, PR China
[b] Affiliated Eye Hospital of Shandong University of Traditional Chinese Medicine, 48#, Yingxiongshan Road, Jinan 250002, PR China
[c] Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Therapy of Ocular Diseases in Universities of Shandong, Eye Institute of Shandong University of Traditional Chinese Medicine, 48#, Yingxiongshan Road, Jinan 250002, PR China

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ABSTRACT

Purpose: The aim of the present study was to develop and optimize sinomenine hydrochloride (SIN) *in situ* gel for uveitis treatment.
Methods: Carbopol 940 was used as the gelling agent in combination with hydroxypropyl methylcellulose (HPMC), which acts as a viscosity enhancer. The formulations were prepared using various concentrations of Carbopol 940 and HPMC. The prepared *in situ* gels were evaluated for gellation, drug release, ocular irritation, elimination time and pharmacokinetic studies. Furthermore, the effect of SIN on the development of experimental autoimmune anterior uveitis (EAAU) was assessed.
Results: The optimum concentration of Carbopol was 0.1% (w/v), and that for HPMC was 0.4% (w/v). Which showed a significant enhancement in gel strength in the physiological condition while free flowing at non-physiological condition. Optimum formula F_{2-3} consisting of 0.5% SIN was prepared and kept as gel group, and 0.5% SIN solution was prepared and kept as control group. Gel group provided sustained release of the drug over a period of 480 min. No evidence of overt toxicity and irritation was observed in any study. The elimination time of control group and gel group was completed within 10 min and 25 min, respectively. The area under the aqueous humor concentration *vs.* time curve (AUC_{0-t}) and maximum concentration (C_{max}) values of gel group was 2.70-fold and 1.79-fold higher than that of control group. Additionally, clinical examination showed that SIN suppressed inflammation in EAAU.
Conclusions: These results support the potential applications of SIN *in situ* gel for uveitis treatment.
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1. Introduction

Uveitis is an inflammatory eye disease characterized by intraocular infiltration of various populations of leukocytes, and is a Th-1 mediated autoimmune disease [1,2]. Uveitis, a common cause of vision loss, accounts for 5% to 15% of all cases of blindness worldwide affecting individuals of all ages, both sexes, and all races [3]. Possible complications of chronic uveitis include glaucoma, cataracts, accumulation of fluids within the retina, retinal detachment and vision loss [4,5]. Therefore, control of inflammation in uveitis is critical to minimize the vision loss. For the treatment of uveitis, non-steroidal anti-inflammatory drugs (NSAIDs), steroidal agents and immunosuppressants are usually used. However, these drugs are known to produce various side effects, such as increased intraocular pressure, cataract and immunodeficiency [6–8]. Efforts need to be made to seek therapeutic agents that can be used for long-term administration. EAAU in rats is an animal model of organ-specific autoimmune inflammatory disease of the eye that bears close resemblance to human idiopathic anterior uveitis [9]. Various investigators have suggested the use of these animal models to study the efficacy of pharmacologic inhibitors in patients [10,11].

For many decades, numerous bioactive products isolated from various natural resources have influenced modern drug discovery across the therapeutic spectrum. Therefore, great efforts have been made to discover lead compound from natural product in an attempt to obtain new anti-inflammatory drugs. The alkaloid sinomenine is a pure compound extracted from the Chinese medicinal plant, *Sinomenium acutum*, which has been utilized to treat inflammatory diseases for many centuries. A vast number of pharmacological and clinical studies performed in China and Japan have demonstrated that the pure alkaloid extract possesses anti-inflammatory and immunoregulatory properties, as well as mild sedative and analgesic actions due to its chemical structure, which is similar to morphine [12–15]. Chemically, sinomenine hydrochloride is 7, 8-didehy-dro-4-hydroxy-3, 7-dimethoxy-17-methyl-(9a, 13a, 14a)-morphinan-6-one (Fig. 1), which has a molecular weight of

* Corresponding author at: Ophthalmology Department, the Affiliated Eye Hospital of Shandong University of Traditional Chinese Medicine, 48#, Yingxiongshan Road, Jinan 250002, PR China. Tel.: +86 531 58859696; fax: +86 531 82861167.
E-mail address: yankeboshi@163.com (H. Bi).

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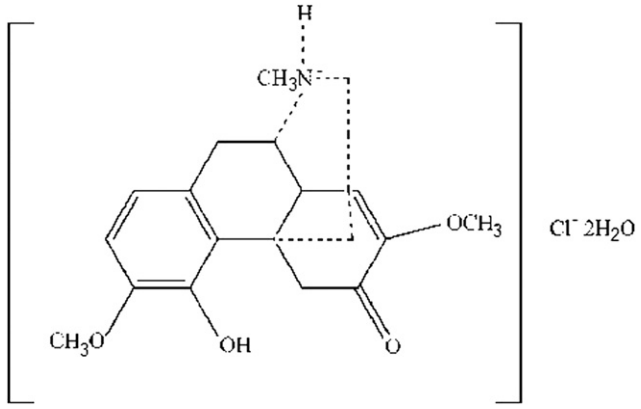

Fig. 1. The molecule structure of SIN.

401.89. As a potent NSAID and immunosuppressive agent, its efficacy has been proven beyond doubt in the treatment of systemic diseases such as rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), as well as endogenous, non-infectious refractory uveitis and other immune-related diseases [16–19]. Based on its potent anti-inflammatory properties, sinomenine was also taken into consideration for the treatment of allogeneic graft rejection and was found to effectively inhibit allograft rejection [20]. However, like other NSAIDs, SIN may cause gastric intestine and kidney damage, and besides may affect liver and heart after long-term treatment [21,22], which makes the topical dosage forms of SIN an attractive alternative delivery route to avoid the oral side effects and provide relatively consistent drug levels for prolonged periods.

The conventional eye drops are eliminated from the precorneal area immediately and only 1–10% of topically applied drug is absorbed [23], which also includes absorption into the gastrointestinal tract due to drainage through the nasal lacrimal duct [24]. The short precorneal contact time combined with corneal impermeability results in low bioavailability, and as a result, frequent instillation of concentrated solutions is needed in order to achieve the desired therapeutic effects [25,26]. In order to increase the effectiveness of the drug a dosage form should be chosen which increases the contact time of the drug in the eye. This may increase the bioavailability, reduce systemic absorption and reduce the need for frequent administration leading to improved patient compliance [27].

Various ophthalmic vehicles such as inserts, ointments, aqueous gels and nanosuspension, have been developed in order to lengthen the residence time of instilled dose and enhance the ophthalmic bioavailability [28,29]. These ocular drug delivery systems, however, have not been used extensively because of some drawbacks such as blurred vision from ointments or low patient compliance from inserts [30]. Several *in situ* gel forming system have been developed to prolong the pre-corneal residence time of a drug and improve ocular bioavailability. Gels of pharmaceutical significance have been prepared by using various types of materials. Carbopol is a polyacrylicacid (PAA) polymer, which shows a sol to gel transition in aqueous solution as the pH is raised above its p_{Ka} of about 5.5 [31]. Carbopols are essentially nontoxic and nonirritant materials with no evidence of their hypersensitivity in human subjects when used topically [32]. However, the concentration of carbopol required to form stiff gel results in highly acidic solutions, which are not easily neutralized by the buffering action of the tear fluid [33]. A reduction in carbopol (anionic polymer) concentration without compromising the gelling capacity and rheological properties of the delivery system was achieved by the addition of viscosity enhancing polymers such as HPMC.

The objective of the present research was to develop a pH-triggered *in situ* gelling system for sustained ophthalmic delivery of SIN. A combination of Carbopol 940 and HPMC was investigated as vehicle for the formulation of eye drops of SIN (0.5%, w/v) that would gel when instilled into the eye, and provide sustained release of SIN during treatment of uveitis and some other ocular inflammation.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Carbopol (940P NF, B. F. Goodrich), HPMC (Methocel K4M) were kindly gifted by Colorcon (UK). SIN (>98%) and fluorescein disodium salt were obtained from Shaanxi Sciphar Biotechnology Co., Ltd (China). Prednisolone acetate eye drops was purchased from YanLiJian Pharmaceutical Co., LTD (Hangzhou, China). Inter-photoreceptor retinoid-binding protein (IRBP) was purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China). Freund's complete adjuvant was purchased from Sigma-Aldrich, St. Louis, MO. All other chemicals and solvents were of analytical grade.

2.1.2. Animals

Female Lewis rats (160–180 g) of specific pathogen-free grade were purchased from Peking Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. White New Zealand rabbits weighing 2.0–3.0 kg and free of any signs of ocular inflammation or gross abnormalities were obtained from the Animal Experimental Center of the Shan-Dong University. All animals were fed and maintained according to the guidelines of Care and Use of Laboratory Animals published by the China National Institute of Health. All experimental procedures adhered to the Association for Research in Vision and Ophthalmology Statement for the use of animals in ophthalmic and vision research.

2.2. Liquid chromatography

SIN content in the samples was quantitated by reversed phase (RP)-HPLC (Agilent 1100, HP Inc., USA; Diamonsil® C_{18} column, 250 mm × 4.6 mm, 5 μm, Dikma Technology Co., China). The column temperature was maintained at 30 °C. The mobile phase consisted of acetonitrile:water:triethylamine (21:79:0.05, v/v) at a flow of 1 ml min^{-1}. Detection was performed at 262 nm. A 20 μl aliquot of the sample solution was loaded onto the column.

2.3. Preparation of pH-triggered gels

2.3.1. Selection of polymer composite

Aqueous solutions of varying concentrations of Carbopol 940 and HPMC (formulation codes F_{1-1}, F_{1-2},..., F_{3-5}) were prepared and evaluated for gelling capacity and viscosity in order to identify the compositions suitable for use as *in situ* gelling systems (Table 1). The gelling capacity was determined by placing 100 μl of the system in a vial containing 2 ml of artificial tear fluid (NaCl 0.670 g, sodium bicarbonate 0.200 g, calcium chloride·2 H_2O 0.008 g, purified water q.s. 100.0 g) freshly prepared and equilibrated at 37 °C and visually assessing gel formation and noting the time for gelation and the time taken for the gel formed to dissolve. Viscosity at 20 rpm was measured using a Brookfield Synchrolectric viscometer (RVT model) in a small volume adapter used for purposes of comparative evaluation. The pH of all the sample solutions was adjusted by 0.1 M triethanolamine solution.

2.3.2. Preparation of pH induced in situ gel

A concentrated Carbopol 940 solution was obtained and HPMC was added and allowed to hydrate. For preparations of SIN-containing polymer solutions, the desired amounts of SIN were added to the Carbopol/HPMC solutions with continuous stirring until thoroughly mixed. Benzalkonium chloride (BKC) was then added and the solutions were filtered through 0.2 mm cellulose acetate membrane filter. The solutions were then brought to volume 100 ml with purified

Table 1
Combination of Carbopol 940 and HPMC (K4M) studied (mean ± SD, n = 3).

Formulations	Contents (%, w/v)		Gelling capacity	Viscocity (mPa s at 20 rpm)		
	Carbopol 940	HPMC (K4M)		pH 5.5 ± 0.2	pH 6.0 ± 0.2	pH 7.0 ± 0.2
F_{1-1}	0.08	0.1	−	100 ± 5	325 ± 7	500 ± 19
F_{1-2}	0.08	0.2	+	140 ± 7	560 ± 14	700 ± 21
F_{1-3}	0.08	0.4	+	255 ± 11	840 ± 15	1000 ± 23
F_{1-4}	0.08	0.6	+++	680 ± 13	1400 ± 31	2800 ± 140
F_{1-5}	0.08	0.8	+++	900 ± 87	3700 ± 107	5400 ± 130
F_{2-1}	0.1	0.1	+	285 ± 23	740 ± 54	1360 ± 98
F_{2-2}	0.1	0.2	+	305 ± 12	980 ± 43	1800 ± 120
F_{2-3}	0.1	0.4	++	700 ± 85	1120 ± 49	4300 ± 120
F_{2-4}	0.1	0.6	+++	840 ± 22	5900 ± 250	7400 ± 310
F_{2-5}	0.1	0.8	+++	1360 ± 145	7100 ± 440	7600 ± 360
F_{3-1}	0.12	0.1	+	305 ± 32	900 ± 55	1100 ± 110
F_{3-2}	0.12	0.2	++	860 ± 105	1580 ± 40	4200 ± 100
F_{3-3}	0.12	0.4	+++	1500 ± 150	3500 ± 97	4400 ± 110
F_{3-4}	0.12	0.6	+++	2400 ± 240	4200 ± 120	5500 ± 220
F_{3-5}	0.12	0.8	+++	2900 ± 250	7800 ± 350	7600 ± 340

Note: −, no gelation; +, gels after a few minutes, dissolves rapidly; ++, gelation immediate, remains for few hours; +++, gelation immediate, remains for extended period.

water and thoroughly agitated while cold. All formulations were allowed to equilibrate for 24 h at room temperature prior to the evaluation both *in vitro* and *in vivo*.

2.4. In vitro release studies

According to previous reports [23], the *in vitro* release test was done using a membrane less dissolution model (4.0 cm i.d. and 0.9 cm in depth) with a dissolution testing apparatus (ZRS-8G, Tianjin, China). A 1 ml volume of the formulation was accurately pipetted into this equipment. The container was immersed in 500 ml freshly prepared STF, which was used as the release medium. The temperature and rotating rate were maintained at 34 ± 1 °C and 50 rpm, respectively. Aliquots (5 ml) were withdrawn from the release mediums at each sampling time and replaced by an equal volume of the release medium. The samples were filtered through 0.45 μm syringe filters, and were subjected to HPLC analysis to determine the SIN concentrations.

2.5. Ocular irritation studies

In this study, both the Draize method and histological examination were used to evaluate the ocular irritation of 0.5% SIN solution and 0.5% SIN *in situ* gel.

2.5.1. Evaluation with the Draize method

Ocular irritation was evaluated according to the Draize method [34]. Rabbits were treated with different ophthalmic formulations (control group or gel group). Draize method ocular irritation scores for every rabbit were calculated by adding the irritation scores for the cornea, iris, and conjunctiva. The eye irritation score was obtained by dividing the total score for all rabbits by the number of rabbits. Irritation was classified according to four grades: non-irritating, score 0–3; slightly irritating, score 4–8; moderately irritating, score 9–12; and severely irritating, score 13–16.

2.5.2. Histological examination

The effects of SIN on corneal structure and integrity were examined *in vitro*. Corneas were removed from the eyes of freshly sacrificed rabbits and incubated at 37 °C for 2 h in 0.5% SIN solution (control group) or 0.5% SIN *in situ* gel (gel group). Sodium dodecylsulfate (SDS) solution in phosphate buffer saline (PBS) 0.1% (*w/w*) was used as the positive control. After incubation, corneas were washed with PBS and immediately fixed in formalin (8%, *w/w*). Tissues were dehydrated in an alcohol gradient, placed in melted paraffin, and solidified in block form. Cross sections (<1 μm) were cut, stained with haematoxylin and eosin (H&E), and microscopically observed for any pathological modifications.

2.6. In vivo elimination study

To reach the goal of studying the elimination time of the gels we dispersed fluorescein disodium salt in the preparations since they were well retained in the gel and easily visualized using a slit lamp. 50 μl of each preparation was added into the lower conjunctival sac of rabbit (n = 3), a slit lamp with blue light was used to monitor the disappearance of the fluorescein disodium salt. At selected time intervals the eyes were inspected, when only a minute amount or none of the gel remained it was considered as lost from the eye. The foregoing time for inspection was defined as the elimination time.

2.7. Ocular pharmacokinetics

In this study, rabbits (n = 3) had been treated with 0.3% (w/v) ofloxacin ophthalmic solution for 4-day before surgery. Pupils were dilated by topical instillation of 0.4% tropicamide prior to the probe implantation. Before the probe implantation, the rabbits were anesthetized with sodium pentobarbital injected through the marginal

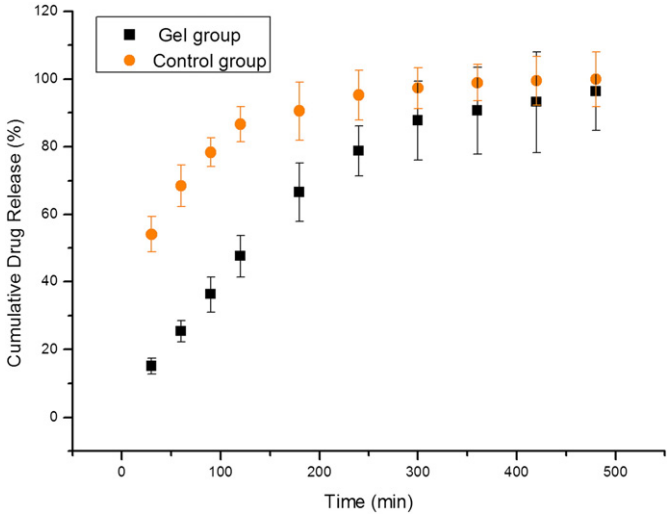

Fig. 2. *In vitro* release study: cumulative amount of SIN released as a function of time from various formulations (mean ± SD, n = 4). Formula F_{2-3} consisting of 0.5% SIN was prepared and kept as the gel group, and 0.5% SIN solution was prepared and kept as the control group.

ear vein and the rabbits were kept under anesthesia throughout the experiment. A custom designed CMA 30 linear microdialysis probe (CMA/AB Microdialysis, Sweden) was implanted into the anterior chamber of each eye as described previously [35]. After probe implantation, the animals were allowed to stabilize for 2 h. And then probes were perfused with IPBS at a flow rate of 2 μl min^{-1} using the CMA 402 syriage pump (CMA/AB Microdialysis, Sweden).

To determine *in vivo* probe calibration, the microdialysis probe was perfused at a rate of 2 μl min^{-1} with different concentrations of standard SIN IPBS solution. Dialysates were collected for 20 min after 30 min of perfusion. The dialysate (20 μl) was injected into the HPLC column and drug levels were determined. Relative recovery is expressed as a ratio of drug concentration in dialysate to that in the solution bathing the dialysis membrane. Recovery (R) is the ratio of drug concentration in the dialysate (C_d) to that in the aqueous humor (C_a), calculated according to the following equation:

$$R_{in\ vivo} = \frac{C_d - C_p}{C_a - C_p}$$

where C_p is the SIN concentration in the perfusate. C_d is the concentration of dialysate and C_a is the concentration in aqueous humor. A linear equation was plotted by ($C_d - C_p$) *vs.* C_p, and the slope of the line gave the recovery ($R_{in\ vivo}$).

The probe was perfused with IPBS at a flow rate of 2 μl min^{-1}. After that, an aliquot (100 μl) of control group or gel group formulation was instilled in the lower cul-de-sac of each eye, and the upper and lower eyelids were gently held closed for 10 s to maximize drug cornea contact. The experiment was continued for 8 h after instillation of the formulation. Samples were collected every 20 min within 2 h or every 30 min during the 3rd hour after instillation. At the end of the experiment, euthanasia was performed under deep anesthesia with an intravenous injection of sodium pentobarbital through the marginal ear vein. Samples were analyzed by HPLC.

Drug concentration in aqueous humor was calculated from relative recovery *in vivo* and drug levels in dialysates. The values of the PK parameters were calculated with noncompartmental analysis by the software program DAS (version 2.0, Chinese Pharmacological Association), including the maximum concentration (C_{max}), time to reach the maximum concentration (T_{max}), area under concentration–time curve (AUC_{0-t}), half-life of elimination ($t_{1/2}$), and mean residence time (MRT).

Table 2
Ocular irritation test results for multiple ophthalmic drug formulations (n = 3).

Formulations	Parameters	Ocular irritation scores					
		1 h	2 h	4 h	24 h	48 h	72 h
Control group	Corneal	Opacity	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctivae	7	6	3	0	0	0
	Average	Score	1.75	1.5	0.75	0	0
Gel group	Corneal	Opacity	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctivae	6	5	3	0	0	0
	Average	Score	1.5	1.25	0.75	0	0

Note: Formula F_{2-3} consisting of 0.5% SIN was prepared and kept as the gel group, and 0.5% SIN solution was prepared and kept as the control group.

2.8. Induction and assessment of EAAU

2.8.1. Induction of EAAU

The peptide was prepared by emulsification of 100 μg IRBP1177–1191 peptide in Freund's complete adjuvant containing 2.5 mg ml^{-1} of mycobacterium tuberculosis H37Ra in a total volume of 0.1 ml. Female Lewis rats were immunized by an injection of 0.1 ml peptide antigen in each footpad.

2.8.2. Clinical examination and assessment

The immunized rats were divided into four groups (blank group, control group, gel group and positive control group) with 5 in each group. They were intervened 3 times per day from the first day after immunization. And 5 unimmunized rats were set as normal control group. Normal control group was treated without any drops. Blank group was

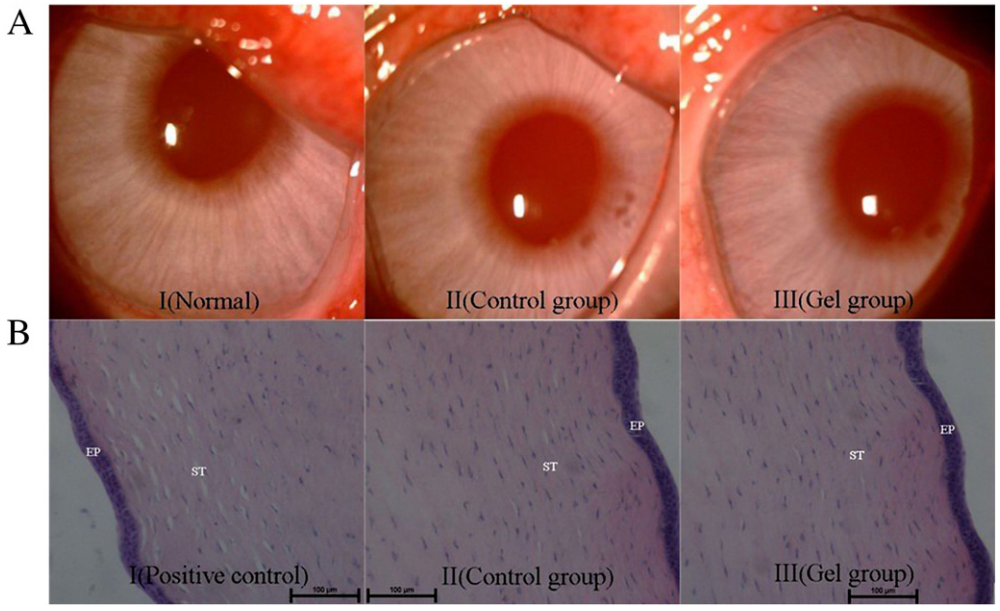

Fig. 3. Ocular irritation was evaluated by both the Draize method and histological examination. (A): Draize method: Rabbits were treated once every 30 min for 6 h (12 times) with different formulations. The ocular condition was recorded and photographed at 1, 2, 4, 24, 48, and 72 h after the last administration. (I) Rabbit eyes were dealt without any treatment; (II) Rabbit eyes were treated with 0.5% SIN eyedrops. (III) Rabbit eyes were treated with 0.5% SIN *in situ* gel. (B): Histological examination: Histological cross sections of excised rabbit cornea demonstrating epithelium (EP) and stroma (ST) stained with hematoxylin–eosin after incubation at 37 °C for 0.5 h. (I) Corneas were incubated with sodium dodecylsulfate (SDS) solution 0.1% (w/w) (positive control); (II) corneas were incubated with 0.5% SIN solution (control group); and (III) corneas were incubated with 0.5% SIN *in situ* gel (gel group). Scale bar = 100 μm.

treated with PBS. Control group was treated with 0.5% SIN eye drops. Gel group was treated with 0.5% SIN *in situ* gel. Positive control group was treated with 1% prednisolone acetate eye drops. The rats were clinically observed on a daily basis with slitlamp biomicroscopy for clinical signs of ocular inflammation and scored according to acknowledged criteria [36]. Severity of EAAU was scored on a scale of 0 (no disease) to 4 (maximum disease). Disease severity was clinically assessed with a scale ranging from 0 to 4: 0 = normal; 1 = slight iris-vessel dilatation and some anterior chamber cells; 2 = Iris hyperemia, with some limitation in pupil dilation, anterior chamber cells, and a slight flare; 3 = a miotic, irregular, hyperaemic, and (sometimes) slightly damaged iris, with a considerable flare and cells (especially with accumulation near the iris); and 4 = a seriously damaged and hyperaemic iris, a miotic pupil often filled with protein, and cloudy gellike aqueous humor.

2.8.3. Eye histopathology

The immunized rats were divided into two groups (blank group, gel group) with 3 in each group. And 3 unimmunized rats were set as normal control group. They were intervened 3 times per day from the first day after immunization. Normal control group was treated without any drops. Blank group was treated with PBS. Gel group was treated with 0.5% SIN *in situ* gel. Rats were sacrificed at day 14 post-immunization (peak of EAAU) and the eyes were enucleated. Freshly enucleated rat eyes were fixed in neutral buffered 10% formalin solution for 24 h at room temperature, dehydrated in ethanol and embedded in paraffin.

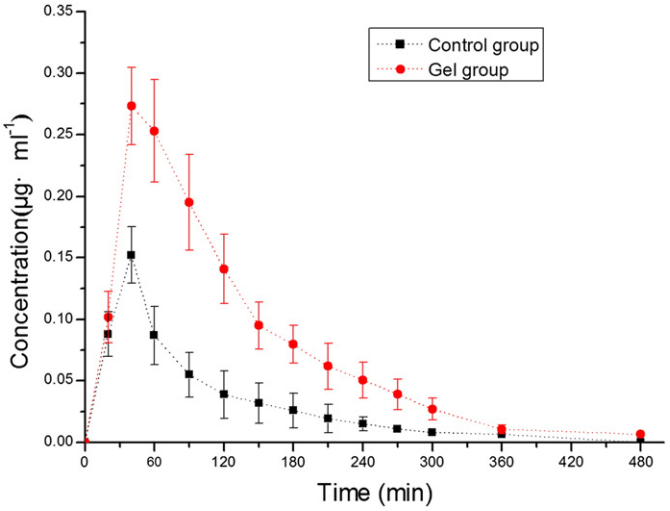

Fig. 5. *In vivo* transcorneal absorption of SIN after ophthalmic administration of 0.5% SIN solution (control group) or 0.5% SIN *in situ* gel (gel group). Values represent mean ± SD. (n = 3).

Then, 5 μm sagittal sections were cured and stained with hematoxylin and eosin (H&E) for histopathologic analysis. Sections were examined using a light microscope (Nikon Eclipse 55i, Japan).

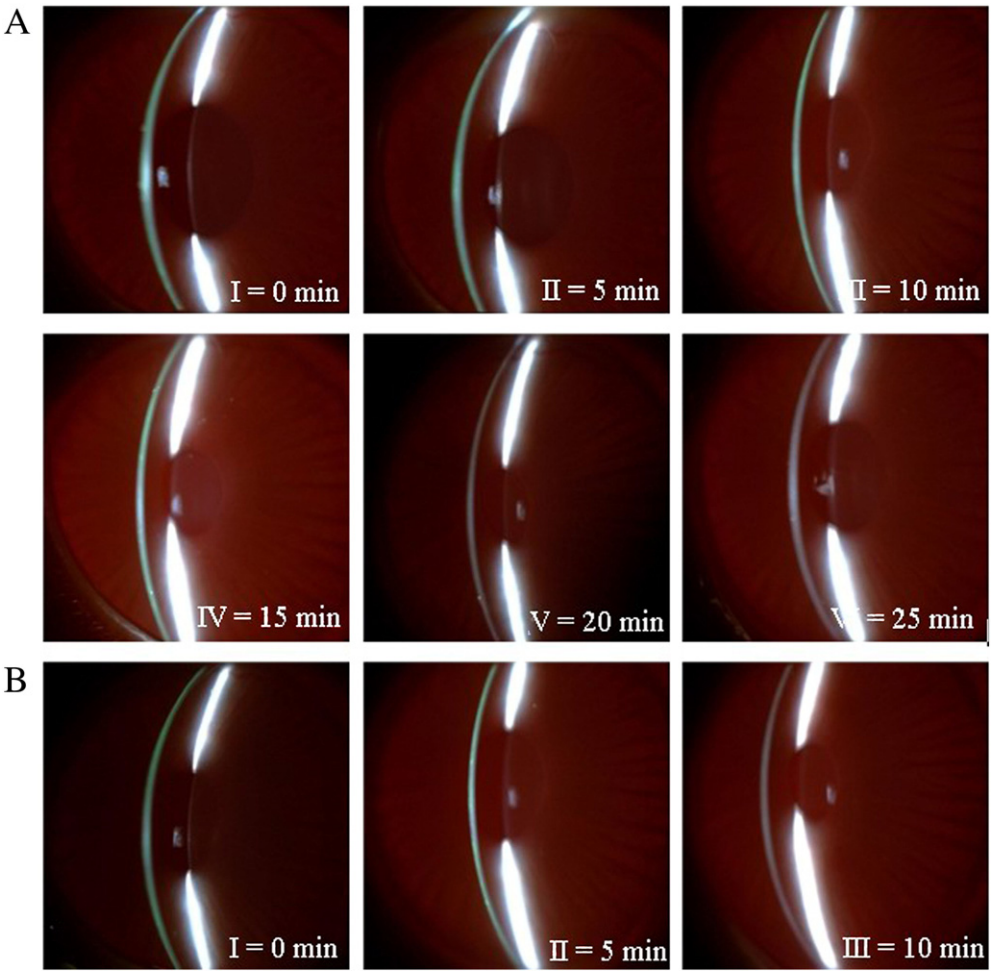

Fig. 4. *In vivo* elimination study. Fluorescein disodium salt was dispersed in the preparations and visualized using a slit lamp. 50 μl of each preparation was added into the lower conjunctival sac of rabbit, a slit lamp with blue light was used to monitor and photograph the disappearance of the fluorescein disodium salt. (A) Elimination time after the gel group was dropped. (I–VI: 0, 5, 10, 15, 20, 25 min). (B) Elimination time after the control group was dropped. (I–III: 0, 5, 10 min). Formula F_{2-3} consisting of 0.5% SIN was prepared and kept as the gel group, and 0.5% SIN solution was prepared and kept as the control group.

Table 3
Pharmacokinetic parameters of SIN in aqueous humor (mean ± SD, n = 3).

Parameters	Control group	Gel group
$AUC_{(0-8)}$ (μg ml^{-1} h)	13.46 ± 2.32	36.27 ± 3.54*
C_{max} (μg ml^{-1})	0.15 ± 0.02	0.27 ± 0.02*
T_{max} (min)	40	40
$t_{1/2}$ (min)	65.94 ± 4.32	81.64 ± 6.61*
$MRT_{(0-8)}$ (h)	100.89 ± 8.33	124.16 ± 7.62*

Note: Formula F_{2-3} consisting of 0.5% SIN was prepared and kept as the gel group, and 0.5% SIN solution was prepared and kept as the control group.
* $P < 0.05$ *vs.* control group.

2.9. Statistical analysis

All the data were processed with SPSS 17.0. All the data were processed for one-way ANOVA followed by post hoc analysis for significance with the LSD-t multiple comparison test. $P < 0.05$ was regarded as statistically significant.

3. Results and discussion

3.1. Selection of vehicle

The use of Carbopol in *in situ* gelling systems is substantiated by the property of its aqueous solutions to transform into stiff gels when the pH is raised [37]. However, the concentration of Carbopol required to form stiff gels results in highly acidic solutions that are not easily neutralized by the buffering action of the tear fluid. A reduction in Carbopol concentration without compromising the gelling capacity and rheological properties of the delivery system may be achieved by the addition of viscosity-enhancing polymers such as HPMC. In order to identify the compositions suitable for use as *in situ* gelling systems, various concentrations of Carbopol and HPMC were prepared and evaluated for gelling capacity. Table 1 shows that grade "++" of gelling capacity was more satisfactory. And 0.1% Carbopol/0.4% HPMC was selected as the developed vehicle for further studies.

3.2. Preparation of formulations

The two main prerequisites of an *in situ* gelling system are viscosity and gelling capacity. The formulation should have an optimum viscosity that will allow easy instillation into the eye as a liquid (drops), which would undergo a rapid sol-to-gel transition (triggered by a rise in pH from 5.5 to 7.0). Additionally, to facilitate sustained release of drug to the ocular tissue, the gel should preserve its integrity without dissolving or eroding for a prolonged period of time. SIN-containing F_{2-3} (0.1% Carbopol/0.4% HPMC) was selected as the developed formulation, which has satisfactory attributes of viscosity, gelling capacity and easy instillation into the eye.

3.3. In vitro drug release studies

The release mechanism *in vitro* was dependent on two simultaneous processes: water migration into the *in situ* gelling system and drug diffusion. The prolonged release may be probably due to the formation of hydrogen bonds between drug and polymers, which have helped in rate control release of drug. *In vitro* drug release studies of optimized formulae F_{2-3} was shown in Fig. 2 by plotting cumulative % drug release *vs.* time. The results of this study revealed that, SIN-containing F_{2-3} released its drug contents, 96.3%, over a period of 480 min.

3.4. Ocular irritation studies

No evidence of overt toxicity was observed in any study, by ophthalmoscopic (Fig. 3A) or histopathological evaluation (Fig. 3B), as indicated by a lack of corneal degeneration, necrosis, inflammation, or edema. Corneas exposed to 0.1% SDS had epithelial (EP) structural damage due to superficial epithelial cell detachment. Neither the structure nor the integrity of the corneas was visibly in control group and gel group. Irritation scores for each formulation group were less than 2.5, indicating that all formulations were well tolerated (Table 2). Taking into consideration that the rabbit eye is more susceptible to irritant substances than the human eye [38], this result would be considered very promising. Ocular damaging/irritant agents are currently identified and evaluated by the Draize rabbit test, which, however, is being criticized on the basis of ethical considerations and unreliable prognosis of human response [39]. Further more studies should be carried out, particularly long-term administration with penetration enhancers should be concerned.

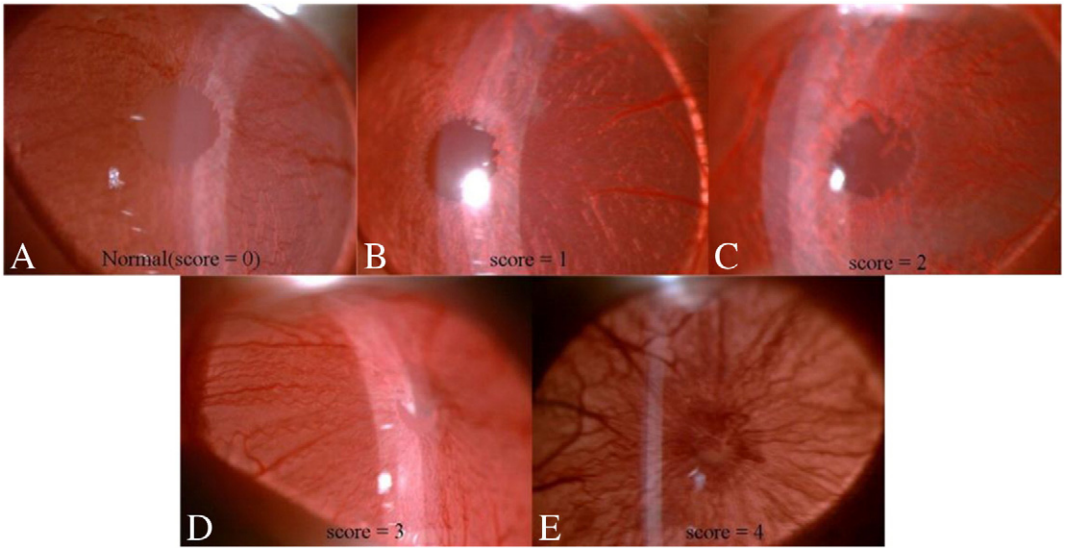

Fig. 6. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease). Disease severity was clinically assessed with a scale ranging from 0 to 4. (A) 0 = normal; (B) 1 = slight iris-vessel dilatation and some anterior chamber cells; (C) 2 = iris hyperemia, with some limitation in pupil dilation, anterior chamber cells, and a slight flare; (D) 3 = a miotic, irregular, hyperaemic, and (sometimes) slightly damaged iris, with a considerable flare and cells (especially with accumulation near the iris); (E) 4 = a seriously damaged and hyperaemic iris, a miotic pupil often filled with protein, and cloudy gellike aqueous humor.

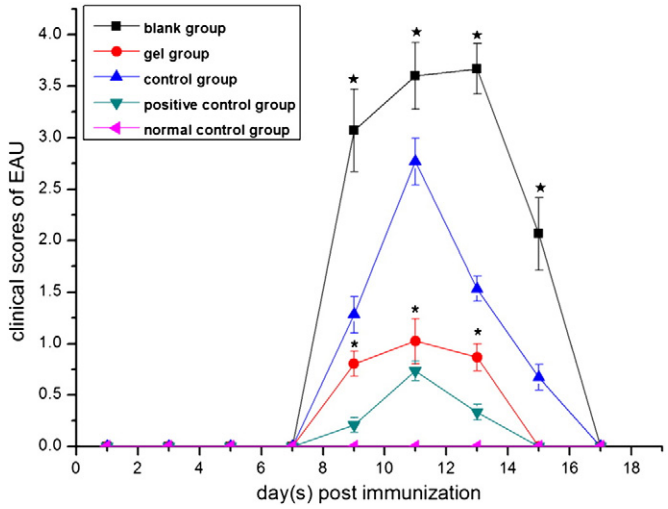

Fig. 7. Clinical scores of the monophasic EAAU (mean ± SD, n = 5). Normal control group was treated without any drops; blank group was dropped with PBS; control group was dropped with 0.5% SIN eye drops; gel group was dropped with 0.5% SIN *in situ* gel; positive control group was dropped with 1% prednisolone acetate eye drops. dpi = day(s) post immunization. Severity of EAAU was scored on a scale of 0 (no disease) to 4 (maximum disease). Disease severity was clinically assessed with a scale ranging from 0 to 4: 0 = normal; 1 = slight iris-vessel dilatation and some anterior chamber cells; 2 = Iris hyperemia, with some limitation in pupil dilation, anterior chamber cells, and a slight flare; 3 = a miotic, irregular, hyperaemic, and (sometimes) slightly damaged iris, with a considerable flare and cells (especially with accumulation near the iris); and 4 = a seriously damaged and hyperaemic iris, a miotic pupil often filled with protein, and cloudy gellike aqueous humor. Data were expressed as mean ± SD. *P < 0.05 *vs.* gel group, ★P < 0.05 *vs.* blank group, one-way ANOVA and followed by Tukey's HSD post-hoc test.

3.5. Elimination studies

The results of precorneal retention study of control group and gel group are shown in Fig. 4. When the preparation was a gel the fluorescein stayed within the gel and gradually disappeared as the gel was disintegrating. For the control group, the behavior of the fluorescein was drastically different, a continuous flow of fluorescein towards the inner canthus along the eyelid margin was observed. This elimination of control group and gel group was completed within 10 min (Fig. 4A) and 25 min (Fig. 4B) in the rabbit eyes, respectively.

In order to increase the effectiveness of the drug a dosage form should be chosen which increases the contact time of the drug in the eye. This may then increase the bioavailability, reduce systemic absorption and reduce the need for frequent administration leading to improved patient compliance. When a drug solution is dropped into the eye, the effective tear drainage and blinking action of the eye result in a 10-fold reduction in the drug concentration within 4–20 min. In this study, the percorneal retention was improved from 10 min to 25 min, then improved local bioavailability, reduced dose concentrations and dosing frequency, and improved patient acceptability, may be achieved.

3.6. Pharmacokinetic profiles

Microdialysis is a technique used to monitor *in vivo* the concentration time course of drugs and endogenous substances in tissue's extracellular fluid [40]. Ocular microdialysis has gained popularity in recent years due to its ability to continuously monitor drug concentrations and substantially reduce the number of animals needed [41–43].

Relative recoveries for SIN estimated in *in vivo* experiments were approximately (20.48 ± 2.02) % (n = 3) measured by retrodialysis. Time–concentration profiles of gel group and control group were shown in Fig. 5 and Table 3. SIN seemed to be eliminated from aqueous humor according to first-order kinetics. Concentrations of SIN in aqueous humor increased rapidly after instillation and reached maximal levels at 40 min in control group. Compared to control group, SIN in gel group exhibited a 2.70-fold greater AUC_{0-t} and 1.79-fold greater C_{max} than that of control group, while T_{max} unchanged.

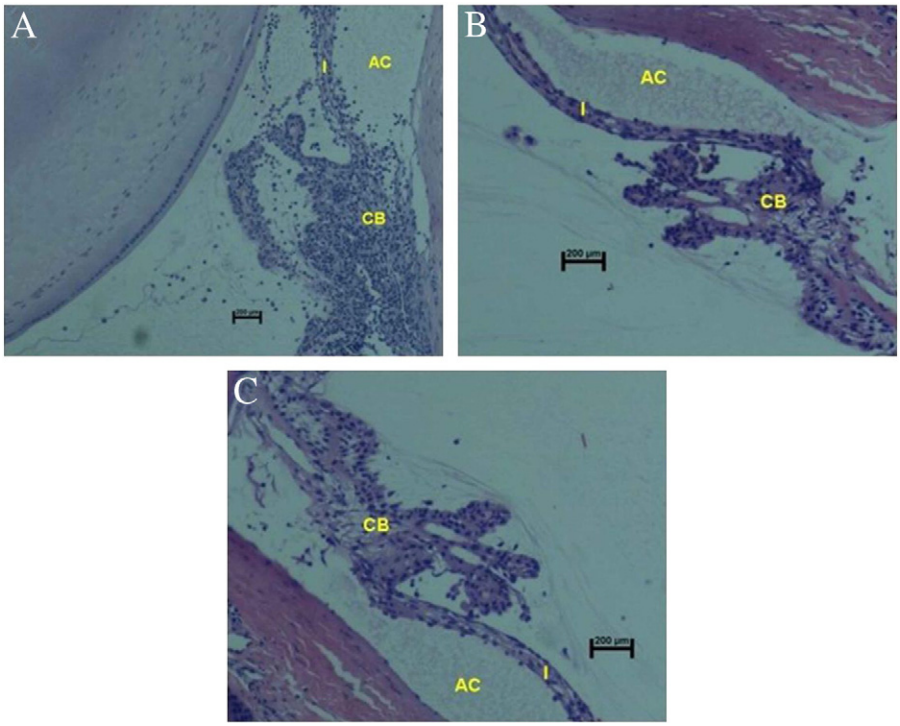

Fig. 8. Histologic changes in the iris (I), the ciliary body (CB) and the anterior chamber (AC) of blank group (A), gel group (B) and normal control group (C). Histopathologic examination of the harvested eyes at day 14 post-immunization demonstrated heavy infiltration of the inflammatory cells, mainly monocytes and T lymphocytes, within the iris (I), the ciliary body (CB) and the anterior chamber (AC) of blank group. In contrast, only few inflammatory cells within the iris/ciliary body and the anterior chamber of gel group. And the iris/ciliary body and the anterior chamber of normal control group showed no infiltration with mononuclear cells. Objective magnification 20×.

3.7. Protective effect of SIN on EAAU

Disease severity was observed daily by slit-lamp microscopy and graded as described in the Materials and methods section (Fig. 6A–E). Based on the clinical course, monophasic EAAU was divided into three stages: an initiation phase from the day of immunization to 8 day post immunization, an effector phase beginning from 8 to 15 day post immunization, with the peak inflammation obtained at 11 day post immunization, and a phase of resolution starting from 15 day post immunization.

The eyes in each group were observed everyday after immunization until day 17 post immunization, and clinical scores were recorded (Fig. 7). The results indicated that gel group and positive control group developed EAAU later and with lighter inflammation than in blank group and control group. The clinical signs of blank group such as dilated or engorged blood vessels in iris were observed on days 8–9 post immunization. The most severe intraocular inflammation was detected on days 13–14 post immunization, as evidenced by an opaque anterior chamber and obscured pupil. On day 15 post immunization, the ocular inflammation was greatly resolved, with only minor clinical signs, and no inflammatory signs were detected on day 17 post immunization. Compared with blank group, there were no inflammatory response on day 9 post immunization and only mild hyperemia of the iris, no hypopyon, normal pupil were observed on day 11 post immunization in the gel group. On day 13 post immunization, the ocular inflammation was almost resolved, with only minor clinical signs. No inflammatory signs were detected on day 15 post immunization. Histopathologic examination of the harvested eyes at day 14 post-immunization demonstrated heavy infiltration of the inflammatory cells, mainly monocytes and T lymphocytes, within the iris (I), the ciliary body (CB) and the anterior chamber (AC) of blank group (Fig. 8A). In contrast, only few inflammatory cells were within the iris/ciliary body and the anterior chamber of the gel group (Fig. 8B). And the iris/ciliary body and the anterior chamber of normal control group showed no infiltration with mononuclear cells (Fig. 8C).

In summary, SIN was successfully formulated in pH-triggered *in situ* gelling system using Carbopol 940 in combination with HPMC K4M. The formulation caused no irritation to rabbit eye tissues. Both the *in vitro* and the *in vivo* results indicated that the *in situ* gel system is a viable alternative to conventional eye drops by virtue of its ability to enhance bioavailability through its longer elimination time and the ability to sustain drug release. More importantly, it was a suitable medium for SIN to treat autoimmune uveitis based on its anti-inflammatory and immunoregulatory characteristics. Additionally, our results demonstrated that the topical administration of SIN *in situ* gel to Lewis rats before the onset of EAAU inhibited disease assessed both clinically and histologically. To our knowledge, this study is the first to demonstrate that SIN can be used to inhibit autoimmune uveitis.

Acknowledgments

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