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Research paper

Bioadhesive sulfacetamide sodium microspheres: Evaluation of their effectiveness in the treatment of bacterial keratitis caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* in a rabbit model

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ABSTRACT

The aim of this study was to prepare bioadhesive sulfacetamide sodium (SA) microspheres to increase their residence time on the ocular surface and to enhance their treatment efficacy on ocular keratitis. Microspheres were fabricated by spray drying method using mixture of polymers such as pectin, polycarbophil and hydroxypropylmethyl cellulose (HPMC) at different ratios. The particle size and distribution, morphological characteristics, thermal behavior, encapsulation efficiency, mucoadhesion and *in vitro* drug release studies on formulations have been investigated. After optimisation studies, SA-loaded polycarbophil microsphere formulation with polymer:drug ratio of 2:1 was found to be the most suitable for ocular application and used in *in vivo* studies. *In vivo* studies were carried out on New Zealand male rabbit eyes with keratitis caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Sterile microsphere suspension in light mineral oil was applied to infected eyes twice a day. Plain SA suspension was used as a positive control. On 3rd and 6th days of the antimicrobial therapy, the eyes were examined in respect to clinical signs of infection (blepharitis, conjunctivitis, iritis, corneal oedema and corneal infiltrates) which are the main symptoms of bacterial keratitis and then cornea samples were counted microbiologically. The rabbit eyes treated with microspheres demonstrated significantly lower clinical scores than those treated with SA alone. A significant decrease in the number of viable bacteria in eyes treated with microspheres was observed in both infection models when compared to those treated with SA alone. In conclusion, *in vitro* and *in vivo* studies showed that SA-loaded microspheres were proven to be highly effective in the treatment of ocular keratitis.

1. Introduction

The eye is a unique organ that is virtually impermeable to most environmental agents. Continuous tear flow, aided by the reflex blinking, provides lubrication to wash away substances from the ocular surface and prevents the accumulation of microorganisms [1]. Tears also contain some antimicrobial agents including lymphocytes, immunoglobulins, lysozyme and lactoferrin which specifically reduce bacterial colonisation on the ocular surface [1]. An intact corneal epithelium provides an effective barrier against most microorganisms and therefore, bacterial keratitis rarely occurs in the normal eye [2]. However, predisposing factors such as corneal injury, allergic hypersensitivity reactions, corneal abnormalities, overuse of contact lenses, complications after laser *in situ* keratomileusis, systemic diseases may alter the defence mechanisms of the ocular surface and permit bacteria to invade the cornea through epithelial defects that occur [2–4].

Bacterial keratitis, due to its potential complications, is one of the most visually threatening ocular infectious pathologies [5,6]. *S. aureus*, a common gram-positive bacterial pathogen implicated in bacterial keratitis, produces many biological active substances and toxins [7]. *P. aeruginosa*, a gram-negative bacterial pathogen, characteristically causes a devastating and rapid keratitis that may lead to corneal thinning and perforation [8,9]. Corneal perforations occur in less than 24 h in the presence of both *P. aeruginosa* and *S. aureus* [10,11]. If appropriate therapy is not promptly initiated, these bacteria can proliferate rapidly through the production of enzymes and exotoxins, and cause a rapidly destructive infection that can lead to loss of the entire eye [12]. Bacterial enzymes such as proteases, coagulases, collagenases and lipases facilitate corneal tissue necrosis and aid in penetration deep into the stroma [13].

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Sulfacetamide sodium (SA), which is a synthetic sulfonamide, has a high antibacterial activity against *S. aureus* and *P. aeruginosa*, and interfere with the synthesis of nucleic acids in sensitive microorganisms by blocking the conversion of *p*-aminobenzoic acid to the coenzyme dihydrofolic acid which is a reduced form of folic acid [14,15]. Eye drops containing 10–30% SA and eye ointments containing up to 10% are used for the treatment of ocular infections [16].

Topical application of drugs into the eye is severely limited by physiological constraints such as tear flow and reflex blinking in the precorneal area resulting in a considerable drug loss. The washout rate reduces the concentration of the drug in a tear film to one-tenth of its starting value in 4–20 min and only a few percent of the administered drug is absorbed by the eye and therefore, duration of the therapeutic action might be quite short [17]. For this reason, standard treatment of severe bacterial keratitis requires administration of drug at frequent intervals.

Different strategies have been developed to increase the drug bioavailability by prolonging the contact time of the formulation with corneal/conjunctival epithelium. Among them, natural and synthetic mucoadhesive polymers interact with the precorneal mucin layer coating the external surface of the eye and show a good potential to increase the bioavailability by increasing the precorneal residence time of the drug [18,19]. Anionic polymers consisting of macromolecular hydrocolloids with numerous hydrophilic functional groups such as carboxyl and hydroxyl also show a good potential to increase the mucoadhesion by forming hydrogen bonds with mucin [20–23].

In addition to mucoadhesion, using the colloidal drug delivery systems such as microparticles [24,25], liposomes [26,27] or nanoparticles [25,28,29] is also desirable for controlling release of the drug. There are many studies about particulate systems prepared with mucoadhesive polymers for increasing precorneal residence time of formulation and optimisation drug release for ocular drug delivery [30,31].

In this study, SA-loaded microspheres were prepared with polycarbophil and pectin with/without hydroxypropylmethyl cellulose (HPMC) using spray drying method. The surface morphology of microspheres, production yield, particle size, actual drug content, encapsulation efficiency, mucoadhesive property and *in vitro* drug release were investigated. Based on *in vitro* evaluation of microspheres, the most suitable formulation chosen for ocular application was examined on rabbits to determine their effectiveness in the treatment of bacterial keratitis caused by either gram-negative organism (*P. aeruginosa*) or gram-positive organism (*S. aureus*). Comparisons were run with SA alone suspension and saline solution.

2. Materials and methods

2.1. Materials

Sulfacetamide sodium (SA) (micronized, mean volume diameter (MVD): 0.81 ± 0.08 μm; polydispersity (span value): 0.214 ± 0.042) was purchased from I.E. Ulagay Company (Turkey), polycarbophil (Noveon AA1) from BF Goodrich (USA); hydroxypropylmethyl cellulose (HPMC) (Methocel® K100MCR Premium EP) from Colorcon (UK); pectin (from citrus fruits, galactronic acid content 93.5%, methoxy content 9.4%) and porcine gastric mucin (Type III, bound sialic acid ~1%) from Sigma (USA); Mueller-Hinton Agar from Bio-Merieux (France). All other chemicals were of analytical grade and used without further purification.

2.2. Fabrication of microspheres

SA-loaded microspheres were prepared at polymer:drug ratios (m/m) of 1:1 and 2:1 by spray drying method. Briefly, polymer solutions in water were prepared with polycarbophil, pectin or their mixtures with HPMC at different ratios and SA was dissolved in polymer solution. The prepared solutions were sprayed through the nozzle of a mini spray dryer (Büchi, Model 191, Switzerland) to obtain microspheres. The process conditions were set as follows: inlet temperature 160 ± 2 °C, outlet temperature 100 ± 2 °C, aspirator setting 100% capacity, pump setting 3 mL min^{-1}, spray flow 600 L h^{-1} and nozzle diameter 0.5 mm. Microspheres were collected and weighed to determine production yield (PY) using Eq. (1):

$$PY(\%) = \frac{\text{practical mass(microspheres)}}{\text{theoretical mass(polymer + drug)}} \times 100 \quad (1)$$

Each microsphere formulation was run in triplicate. Blank microspheres were prepared as negative controls. Formulation parameters of SA-loaded microspheres were summarized in Table 1.

2.3. Determination of particle size

Particle size analyses were carried out on SA-loaded microspheres suspended in absolute ethanol by laser diffraction analyzer (Malvern Instruments, Mastersizer® 2000, UK). Analyses were done in triplicate and the results were expressed in terms of the mean volume diameter (MVD). The polydispersity of microspheres was defined from span value which was calculated using Eq. (2):

Table 1
The production yield, actual drug content, encapsulation efficiency, and particle size and distribution of SA-loaded microspheres (n = 3).

Formulation	SA amount (g/batch)	Pectin amount (g/batch)	HPMC amount (g/batch)	Polycarbophil amount (g/batch)	Actual drug content (% ± SD)	Encapsulation efficiency (% ± SD)	Yield (% ± SD)	Mean particle diameter (μm ± SD)	Span ± SD
PE1	5	5.00	–	–	50.8 ± 0.4	101.6 ± 1.7	48.5 ± 1.5	5.32 ± 0.37	1.79 ± 0.34
PE2	5	2.50	2.50	–	49.4 ± 0.3	98.9 ± 1.1	38.0 ± 2.0	15.39 ± 0.17	3.08 ± 0.67
PE3	5	1.67	3.33	–	51.2 ± 0.7	102.3 ± 1.1	26.6 ± 3.3	19.13 ± 0.44	6.96 ± 0.98
PE4	5	3.33	1.67	–	49.0 ± 0.3	97.9 ± 1.8	40.1 ± 1.1	5.39 ± 0.06	3.01 ± 0.45
PE5	2.5	5.00	–	–	32.8 ± 1.5	98.3 ± 1.4	47.7 ± 1.4	4.65 ± 0.68	1.97 ± 0.25
PE6	2.5	2.50	2.50	–	31.8 ± 0.7	95.3 ± 1.6	38.3 ± 2.5	7.01 ± 0.40	4.66 ± 0.55
PE7	2.5	1.67	3.33	–	32.5 ± 1.4	97.6 ± 1.3	24.7 ± 1.7	7.71 ± 0.25	6.53 ± 0.88
PE8	2.5	3.33	1.67	–	32.6 ± 0.6	97.8 ± 1.3	41.4 ± 3.4	6.07 ± 0.32	3.85 ± 0.55
PO1	5	–	–	5.00	49.9 ± 0.5	99.8 ± 1.0	62.4 ± 2.1	3.18 ± 0.46	1.89 ± 0.21
PO2	5	–	2.50	2.50	50.4 ± 0.3	100.7 ± 0.9	44.6 ± 3.1	6.46 ± 0.74	2.84 ± 0.25
PO3	5	–	3.33	1.67	50.7 ± 1.0	101.3 ± 1.5	36.5 ± 2.7	10.71 ± 0.99	4.35 ± 0.73
PO4	5	–	1.67	3.33	51.5 ± 0.8	103.1 ± 1.8	53.5 ± 3.2	3.48 ± 0.39	2.13 ± 0.42
PO5	2.5	–	-	5.00	33.2 ± 0.4	99.7 ± 1.5	62.7 ± 2.2	1.87 ± 0.04	1.23 ± 0.12
PO6	2.5	–	2.50	2.50	32.7 ± 0.6	98.0 ± 0.9	44.1 ± 1.4	3.14 ± 0.02	3.05 ± 0.36
PO7	2.5	–	3.33	1.67	32.2 ± 0.9	96.6 ± 1.9	34.8 ± 2.4	4.41 ± 0.10	4.14 ± 0.52
PO8	2.5	–	1.67	3.33	32.7 ± 1.0	98.0 ± 1.5	51.3 ± 2.1	2.73 ± 0.02	2.89 ± 0.29

$$Span = \frac{d_{90} - d_{10}}{d_{50}} \times 100 \quad (2)$$

where d_{90}, d_{10}, and d_{50} are the mean diameters at the 90%, 10%, and 50% of the population distribution, respectively.

2.4. Scanning electron microscopy (SEM)

SEM analysis of microspheres was carried out using scanning electron microscope (JEOL JXA 840A, Japan) with an accelerating voltage of 25 kV at a magnification of 2000×. The samples were coated with gold under an argon atmosphere at room temperature yielding a film thickness of 5 nm.

2.5. Actual drug content and encapsulation efficiency

Ten milligrams of microspheres were dissolved in 25 mL phosphate buffer, saline (PBS), pH 7.4, by ultrasonication for 3 h at 25 °C. The samples were filtered through 0.2 μm membrane filter and absorbance of samples was measured at 257 nm using spectrophotometer (UV-1601, Shimadzu, Japan). Actual drug content (AC) and encapsulation efficiency (EE) were calculated using Eqs. (3) and (4), respectively:

$$AC(\%) = \frac{M_{act}}{M_{ms}} \times 100 \quad (3)$$

$$EE(\%) = \frac{M_{act}}{M_{the}} \times 100 \quad (4)$$

where M_{act} is the actual SA content in microspheres, M_{ms} is the weighed quantity of microspheres and M_{the} is the theoretical amount of SA in microspheres calculated from the quantity added in the process. All analyses were carried out in triplicate.

2.6. Mucoadhesion studies

The mucoadhesion of microspheres was measured using a previously described method with some modifications [32]. Measurements were conducted using TA-XT*Plus* Texture analyzer (Stable Micro Systems, Haslemere, Surrey, UK) equipped with a 5 kg load cell and bioadhesion test rig. Cellulose acetate membrane filter (0.22 μm, Millipore Inc., USA) was fitted on the bioadhesion test rig and hydrated with 50 μL 8% porcine gastric mucin (Type III) in simulated lachrymal fluid before the experiment. Microspheres (10 mg) were attached to the lower surface of the probe (P10 perspex, θ: 10 mm, contact area: 0.785 cm^2) of the instrument with double-sided adhesive tape. The tests were conducted at 37 °C. The probe lowered onto the surface of the membrane at a constant speed of 3 mm s^{-1} and a contact force of 3 N applied. After 3 min of contact time, the probe was moved vertically upwards at a constant speed of 3 mm s^{-1}. Work of adhesion (mJ cm^{-2}) and peak detachment force (N) were calculated from force-distance plot using Texture Exponent 4.0.4.0 software package of the instrument. Each experiment was carried out in triplicate.

2.7. In vitro drug release

In vitro release profiles of SA from microspheres were examined in pH 7.4 simulated lachrymal fluid (8.3 g NaCl, 0.084 g $CaCl_2 \cdot H_2O$, 1.4 g KCl, distilled water q.s. 1000 mL) [33]. Ten milligrams of SA-loaded microspheres were placed in separate eppendorf tubes and 1.5 mL simulated lachrymal fluid was added into each tube. The tubes were shaken at 175 ± 5 rpm in a thermostat-controlled Forma orbital shaker (Thermo Electron Corporation, USA) at 37 ± 1 °C. At scheduled time intervals, the tubes were centrifuged at optimum conditions (5000 rpm, 2 min). Optimum centrifugation speed and time which could separate the supernatant without causing microsphere aggregation were determined by preliminary studies. Ten microlitre samples were withdrawn and replaced with fresh medium. Quantification of drug was performed spectrophotometrically at 257 nm. The analyses were carried out in triplicate.

2.8. Sulfacetamide sodium activity against bacterial strains

In order to evaluate the effectiveness of SA against *S. aureus* (ATCC 6538) and *P. aeruginosa* (NCTC 6749) strains which were used in this study to constitute bacterial keratitis, the antibiotic susceptibility of each bacterium was determined. Overnight cultures of the respective bacteria were adjusted to 3×10^8 colony forming units per mL (CFU mL^{-1}) and inoculated on Petri dishes (9 cm) containing Mueller Hinton Agar. The discs (Ø 6 mm) impregnated with 10 μL of 10% SA suspension in light mineral oil were then applied. Petri dishes were incubated at 35 ± 0.1 °C for 24 h. The average diameters of the inhibition zone surrounding the discs were measured. The experiments were carried out in triplicate.

2.9. In vivo studies

Five months old male New Zealand rabbits of 2.8–3.5 kg were used for animal studies. All experiments were conducted according to "Guide for the Care and Use of Laboratory Animals" [34]. The animals were housed in standard cages and kept in a light-controlled room at 19 ± 1 °C and 50 ± 5% relative humidity without any restriction of food or water. The protocol was approved by the Ethical Committee of Cerrahpasa Medical Faculty, Istanbul University, and the experiments were carried out under veterinary supervision.

Ten milligrams of pure drug and selected microspheres (PO5) corresponding to 10 mg of SA were suspended in light mineral oil to make the final concentration of 10% m/m. Light mineral oil was selected as a vehicle to maintain the integrity of particles in formulation before *in vivo* application. Suspensions were placed in vials, sealed and γ-irradiated (25 kGy) using ^{60}Co as the radiation source at Gamma-Pak Sterilization Company, Turkey. The sterility of microspheres was checked according to USP 30 "sterility tests" monograph (2007). The sterile microspheres were kept in sealed vials until experiment.

S. aureus (ATCC 6538) and *P. aeruginosa* (NCTC 6749) which were proven to be sensitive to SA by preliminary studies were chosen for *in vivo* experiments and were diluted to 2000 and 1000 CFU mL^{-1}, respectively, in sterile PBS, pH 7.4, to constitute corneal infection [34,35]. Eighteen animals were divided into three groups. First and second groups were infected with *P. aeruginosa* ($n = 6$) and *S. aureus* ($n = 6$), respectively. Six animals were separated for control (while left eyes of rabbits were infected with *P. aeruginosa,* right ones with *S. aureus*). For this purpose, corneas of each rabbit were intrastromally injected with 0.1 mL bacterial suspension using a 30-gauge needle after local anaesthesia with proparacaine HCl (Alcaine®, Alcon) [35]. Application of bacterial suspension into cornea is as shown in Fig. 1. After 6 h of inoculation, bacterial keratitis was confirmed and topical therapy was started. Left eyes of rabbits were treated with SA alone suspension, while PO5 formulation (microsphere suspension) was applied to right eyes. Treatment consisted of two drops in each eye every 12 h for 6 days (total 12 doses).

On 3rd and 6th days of topical therapy, 1 h after the final dose, the eyes of rabbits from each group were examined using a slit-lamp for clinical signs of bacterial keratitis (blepharitis, iritis, conjunctivitis, corneal oedema, and corneal infiltrates) [36]. Each item was graded using severity scale of 0–3 and total score was calculated for each eye. Treatment and control group animals were then euthanized with intramuscular injections of ketamine (50 mg/kg,

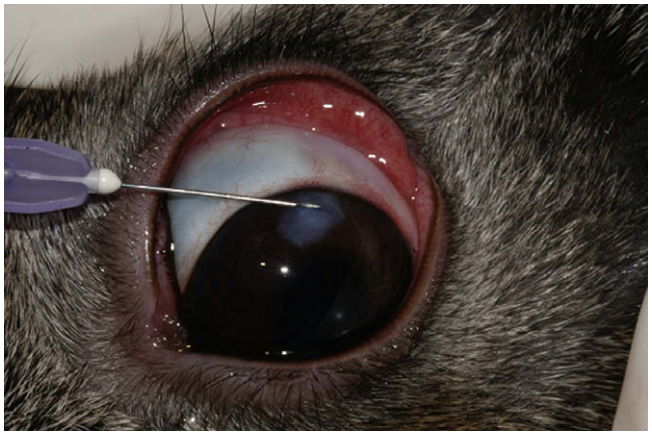

Fig. 1. Intrastromal injection of bacterial suspension into the rabbit cornea to constitute bacterial keratitis using a 30-gauge needle after local anaesthesia with proparacaine HCl.

Ketalar®, Eczacıbaşı) and xylazine (5 mg/kg, Rompun®, Bayer). The corneas were aseptically removed, transferred into tubes containing 1 mL sterile PBS and placed on ice. The corneal buttons were homogenized at 35,000 rpm for 2 min using a high-speed homogenizer (Miccra D-1, Art-Moderne Labortechnik e.K., Germany). The samples were plated on Mueller Hinton agar plates containing 5% sheep blood (BioMerieux, France) and incubated at 35 ± 0.1 °C for 24 h. The number of viable bacteria was determined by the enumeration of colonies formed on plates [7,36].

2.10. Statistical analysis

In vitro and *in vivo* results were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparisons test. Differences between groups were determined for significance by the Chi-square test for the clinical scores. $P < 0.05$ was considered to be the indicative of significance.

3. Results and discussion

3.1. Characteristics of microspheres

The spray drying technique is an easy, reproducible and suitable method to obtain microspheres. This technique provides high encapsulation efficiency, narrow particle size distribution and low level of toxic residual organic solvent when compared to other encapsulation methods [37]. In this study, SA-loaded microspheres with different polymer:drug ratios (1:1 and 2:1) were fabricated using pectin, HPMC, polycarbophil and their mixtures by spray drying method. The production yield, actual drug content, encapsulation efficiency, particle size and distribution of microspheres are shown in Table 1.

The production yields of microspheres were relatively low and ranged between 24.7% and 48.5% for formulations prepared using pectin and pectin–HPMC mixtures, and 34.8–62.7% for formulations prepared using polycarbophil and polycarbophil–HPMC mixtures. The production yield of microspheres containing polycarbophil was higher than those containing pectin. Highest production yield was obtained with formulation containing polycarbophil alone (PO5 = 62.7%). While the addition of HPMC has significantly decreased the production yield of both pectin and polycarbophil microspheres ($P < 0.05$), polymer:drug ratio had no effect on the production yield ($P > 0.05$). This low production yield can be attributed to the low quantity of materials used for spray drying (10 g), to the loss of the smaller particles through the exhaust of the spray-dryer during the manufacturing process [38] and to the adherence of some liquid droplets extensively inside the glass wall of cyclone [37]. The reason for lower production yield of microspheres containing HPMC could also be related to the higher viscosity of feeding solution tending to reduce the spray nozzle efficiency and higher moisture content of prepared particles resulting in the loss of product in a film-like deposit that forms on the cyclone wall because of the high molecular weight and more hydrophilic structure of polymer. However, the loss during spray drying can be reduced significantly if the sample size increases in industrial or large scale operation.

Microparticulate systems for ophthalmic use must be extremely small-sized (smaller than 10 μm) and must have narrow particle size distribution to avoid ocular damage due to abrasion and irritation [29]. In order to compare the particle size distribution results of microspheres prepared in this study, the mean volume diameter (MVD) and span values were determined by laser diffraction analysis. MVD values of microspheres were found to be between 1.87 and 19.13 μm as seen in Table 1. It was found that the particle size of pectin-based microspheres was bigger than polycarbophil-based ones. The size distribution (Span value) of microspheres prepared with plain pectin or plain polycarbophil was narrow and polymer:drug ratio of microspheres did not significantly affect the size distribution ($P > 0.05$). Increasing amounts of HPMC in formulations caused a significant increase in particle size of both pectin ($P < 0.05$) and polycarbophil ($P < 0.05$) microspheres and also significantly widened their size distributions ($P < 0.05$). Similar results were previously obtained by Orhan et al. [39]. In their study, ciprofloxacin hydrochloride-loaded pectin microspheres, which have small particle size (~4 μm) and narrow size distribution, were prepared by spray drying method. Vidgren et al. [40] also reported that the arithmetic mean diameter of polyacrylic acid microspheres containing disodium cromoglycate prepared by spray drying technique ranged between 3.2 and 5.7 μm.

SEM micrographs of SA-loaded microspheres are presented in Fig. 2. Pectin and pectin–HPMC microspheres showed irregular-shape (Fig. 2a), but polycarbophil and polycarbophil–HPMC microspheres were more spherical than pectin-based microspheres, and had porous surface and roughness. Particle morphology and polydispersity of both pectin- and polycarbophil-based microspheres were significantly affected by HPMC content in microspheres. HPMC increased the aggregation of the particles. Different polymer:drug ratios did not substantially influence the morphological characteristics of the spray-dried particles. These results were in agreement with those reported in the literature [39,41].

The drug encapsulation efficiencies were very close to the theoretical values (between 97% and 103%). The actual drug content was found between 49.0–51.2% and 31.8–32.8% for pectin-based microspheres (PE1-PE8) with polymer:drug ratios of 1:1 (PE1-PE4) and 2:1 (PE5-PE8), respectively. Similar results were obtained with polycarbophil-based microspheres and the actual drug content ranged between 49.9–51.5% (PO1-PO4) and 32.2–33.2% (PO5-PO8) at the same polymer:drug ratios indicated above. Polymer type did not influence the actual drug content and encapsulation efficiency ($P > 0.05$). Preparation method also showed good reproducibility, as indicated by analyses of encapsulation efficiency and actual drug content carried out on each group of three batches prepared under identical conditions. All these results demonstrated that spray drying is a suitable technique for the preparation of SA microspheres with appropriate size and high encapsulation efficiency.

3.2. Mucoadhesion studies

Microspheres provide controlled drug release and prolonged therapeutic effect. But without bioadhesion, particles could be

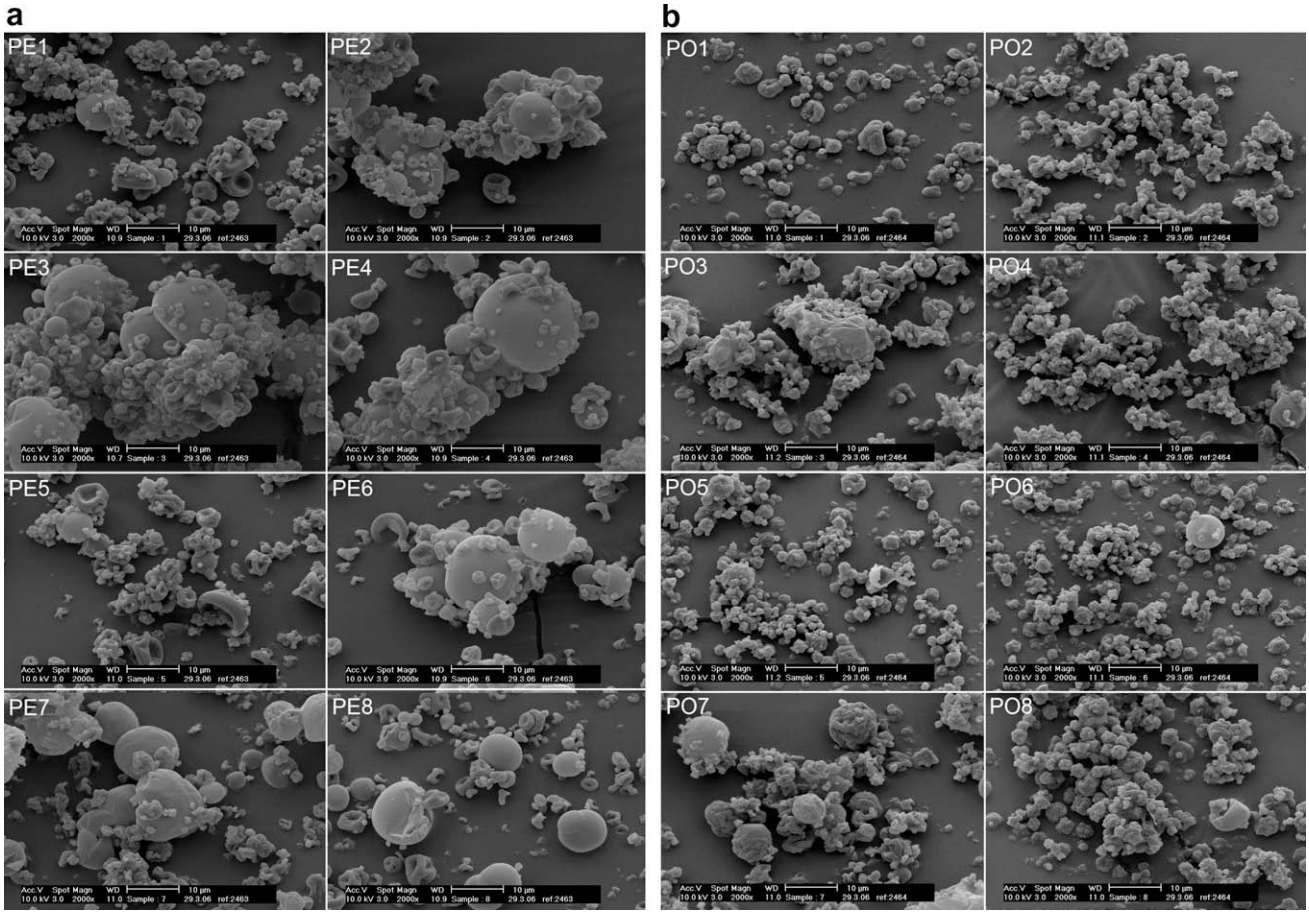

Fig. 2. Scanning electron micrographs of (a) SA-loaded pectin-based microspheres (b) SA-loaded polycarbophil-based microspheres prepared by spray drying method (accelerating voltage of 25 kV; magnification 2000×).

eliminated as quickly as aqueous solutions from precorneal site [42,43]. It is highly desirable to formulate the particles with bioadhesive materials to enhance the retention time in the ocular area. Mucoadhesive polymers influence the corneal retention of microspheres as well as ocular bioavailability from formulations [30,31].

In our study, natural (pectin), semi-synthetic (HPMC) and synthetic (polycarbophil) mucoadhesive polymers were used for the fabrication of microspheres. Mucoadhesion test was performed to measure the adhesive strength of microspheres in simulated conditions by TA-XT*Plus* Texture analyzer. Mucoadhesion test results (work of mucoadhesion and peak detachment force) of microspheres are given in Table 2. Ocular bioavailability from mucoadhesive formulations depends on the polymer's mucoadhesion properties which are affected by its swelling property, hydration time, molecular weight, functional groups (such as carboxyl, amino and sulfate), molecular conformation or chain flexibility and mobility, and concentration [29,44]. At physiological conditions (pH 7.4), mucin is negatively charged owing to the presence of sialic acid groups at the terminal ends of the mucopolysaccharide chains [45]. Generally, neutral polymers such as HPMC undergo swelling in water, which permits entanglement of the polymer chains with mucin [46]. However, many high molecular weight polymers with different functional groups such as carboxyl (polycarbophil), hydroxyl (pectin) and amino (chitosan) have the ability to form hydrogen bonds with mucin molecules [21–23]. These charged polymers (both anionic and cationic) demonstrate a better mucoadhesive capacity in comparison to non-ionic cellulose derivatives and increase the precorneal retention time of formulations. Giunchedi et al. [38] evaluated the precorneal retention time of piroxicam-loaded pectin microspheres *in vivo* in albino rabbits and it was observed that microspheres showed a significantly increased residence time in the eye and improved bioavailability of piroxicam when compared to the commercial piroxicam eye drops. In the same study, the precorneal retention time of fluorescein-loaded pectin particles increased from 0.5 to 2.5 h when compared with a fluorescein solution. Lehr et al. [47] demonstrated that the non-neutralized polycarbophil prolonged the precorneal residence time due to in situ gel formation and mucoadhesion.

Table 2
Work of mucoadhesion and peak detachment force values of SA-loaded microspheres ($n = 3$).

Formulation	Work of mucoadhesion ($\mu J\ cm^{-2} \pm SD$)	Peak detachment force ($N \pm SD$)
PE1	540 ± 30	5.23 ± 0.84
PE2	1830 ± 50	13.16 ± 0.52
PE3	4050 ± 430	23.95 ± 1.07
PE4	1220 ± 70	10.23 ± 0.34
PE5	880 ± 80	8.34 ± 0.12
PE6	2550 ± 230	15.22 ± 0.63
PE7	4580 ± 70	24.99 ± 1.21
PE8	1600 ± 110	11.33 ± 0.19
PO1	8210 ± 240	35.59 ± 1.18
PO2	7030 ± 270	34.59 ± 1.54
PO3	5210 ± 390	27.63 ± 0.68
PO4	7750 ± 560	34.62 ± 1.71
PO5	9710 ± 390	42.62 ± 0.05
PO6	7310 ± 250	36.47 ± 0.75
PO7	6210 ± 170	31.96 ± 0.97
PO8	9470 ± 340	41.21 ± 0.50

In this study, mucoadhesion of microspheres was affected by the nature of the polymer as seen in Table 2. The work of adhesion and peak detachment force of plain pectin microspheres (WA_{PE1}: 540 ± 30 μJ cm^{-2}; PD_{PE1}: 5.23 ± 0.84 N) with polymer:drug ratio of 1:1 were found to be 15 and 7 times less than those of plain polycarbophil microspheres (WA_{PO1}: 8210 ± 240 μJ cm^{-2}; PD_{PO1}: 35.59 ± 1.18 N), respectively, at same polymer:drug ratio. It was previously reported that polycarbophil demonstrates better ocular mucoadhesive capacity than pectin due to existence of non-ionised carboxylic acid groups in the network of polycarbophil [44]. The highest work of adhesion (WA_{PO5}: 9710 ± 390 μJ cm^{-2}) and peak detachment force (PD_{PO5}: 42.62 ± 0.05 N) were recorded in the formulation prepared using polycarbophil alone at a polymer:drug ratio of 2:1. The work of adhesion and peak detachment force of pectin formulations were gradually increased with increasing amounts of HPMC, whereas HPMC addition decreased the bioadhesion of polycarbophil microspheres. It was reported that fast hydration of the polymer affects the bioadhesive force and allow maximal interaction with mucin [44]. We are expecting that HPMC (viscosity of 2% solution in water was 80,000–120,000 cps) used in this study, which was of a fast hydrating-controlled release grade, enhanced the hydration rate of pectin and entanglement of the polymer chains with mucin, and increased mucoadhesion of pectin microspheres. On the contrary, absence of a proton-donating carboxylic group in the structure of non-ionic HPMC reduced the ability of polycarbophil-based microspheres for formation of hydrogen bonds and decreased the mucoadhesion.

3.3. In vitro drug release

Microspheres should enable sustained release of the drug after eye application, maintaining the antibacterial activity during the treatment period. *In vitro* SA release profiles from drug-loaded microspheres are presented in Fig. 3. In our study, the type of the polymer affected the release characteristics of microspheres. Two phases of drug release were observed in the drug release profiles of SA loaded microspheres. The release behavior of microspheres prepared with pectin and pectin–HPMC mixture exhibited higher initial burst release within the first 5 min (between 33% and 51%) compared to microspheres prepared with polycarbophil and polycarbophil-HPMC mixture (between 15% and 30%). The dissolution rate of SA from polycarbophil-based microspheres was slower than the release rate of the drug from pectin-based microspheres. Microsphere production parameters employed such as polymer–drug ratio and HPMC amount in microspheres affected the release characteristics of the drug from formulations. Plain polycarbophil microspheres prepared at a polymer:drug ratio of 2:1 (PO5) showed the slowest dissolution profile (71% of the drug was released after 9 h), whereas increasing amounts of HPMC in microspheres accelerated the drug release from all formulations. Drug release rates were also decreased as polymer:drug ratio was increased, but these differences were not significant for all formulations ($P > 0.05$).

The formulation, PO5, which had the highest production yield (62.7%), exhibited lower initial burst release within the first 5 min (15.25%) and prolonged slow release rate of SA (71% of the drug was released after 9 h) has been selected for the *in vivo* studies.

3.4. In vivo studies

Bacterial keratitis is an ocular emergency that requires immediate and appropriate treatment to limit corneal morbidity and vision loss [1]. Formulation used for the treatment of bacterial keratitis should have a long tear retention time and be able to give a gradual and prolonged release of the antibiotic. Numerous natural and synthetic mucoadhesive polymers were used to increase the viscosity of the preparation and to improve the therapeutic efficacy. However, a number of *in vivo* studies demonstrated that the viscous polymer solutions elicit discomfort, blurred vision, lachrymation and consequently a higher drainage rate and lower bioavailability [48–50]. Particulate systems have potential to become promising systems for ophthalmic drug delivery. Microsphere technology introduces the advantage of superior patient acceptability in combination with extended drug release and improved patient compliance. Microparticles, which are prepared or coated with mucoadhesive polymers, also enhance adherence to the corneal/conjunctival surface as well as therapeutic efficacy [29].

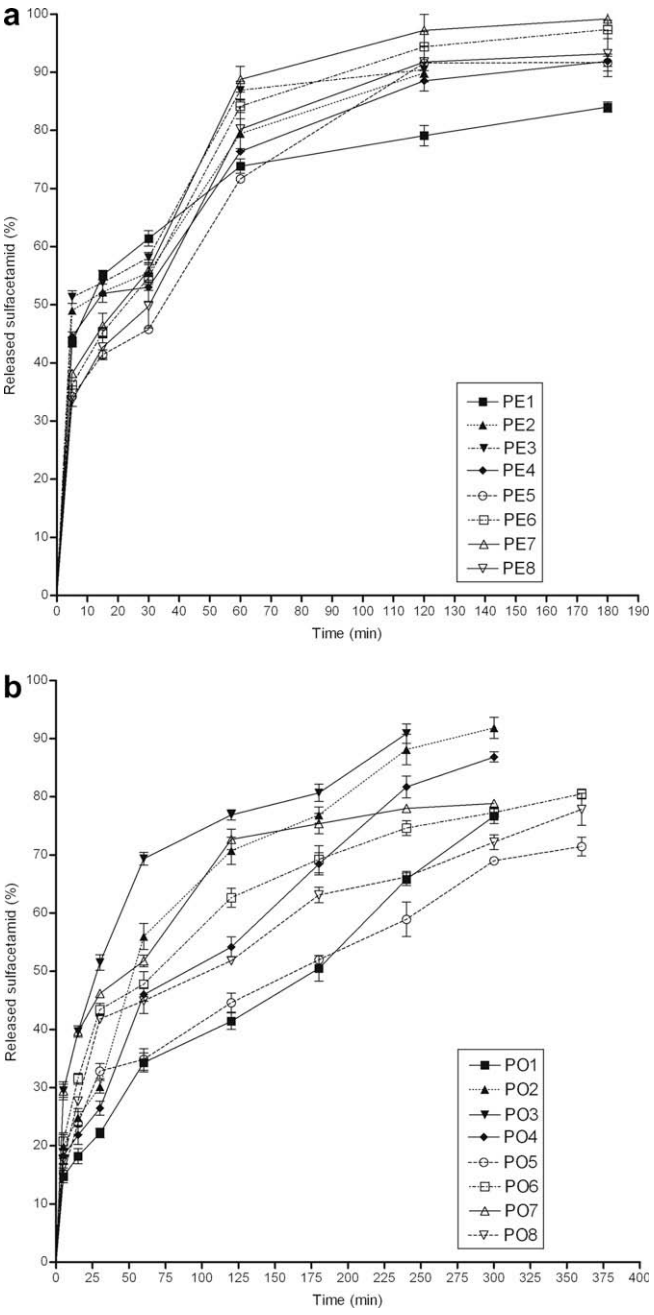

Fig. 3. *In vitro* release profile of SA from (a) pectin and pectin–HPMC microspheres (PE1-PE8), (b) polycarbophil and polycarbophil–HPMC microspheres (PO1-PO8) ($n = 3$).

Our preliminary microbiological studies confirmed that the SA was effective against *S. aureus* and *P. aeruginosa* which were used in this study to constitute bacterial keratitis in eyes (data were not given).

Corneal colony counts for each treatment group of rabbits are shown in Table 3 and graphed in Fig. 4. The results of this study demonstrated that the therapy with SA-loaded bioadhesive microspheres (PO5) was able to successfully treat the bacterial keratitis. Specifically, treatment with microspheres significantly reduced the number of viable *S. aureus* and *P. aeruginosa* recovered and significantly reduced the signs of infection caused by both bacteria. In the *S. aureus* keratitis study, it was observed that the microspheres (1.85 ± 0.15 Log_{10} CFU mL^{-1}) were significantly more effective than SA alone (2.85 ± 0.15 Log_{10} CFU mL^{-1}) ($P < 0.001$) and control group (3.24 ± 0.06 Log_{10} CFU mL^{-1}) ($P < 0.001$) after 3 days treatment. In the case of rabbits infected with *P. aeruginosa*, microsphere formulation also showed significant decrease in colony counts (1.66 ± 0.58 Log_{10} CFU mL^{-1}) compared to pure drug (2.68 ± 0.35 Log_{10} CFU mL^{-1}) ($P < 0.05$) and control group (3.67 ± 0.35 Log_{10} CFU mL^{-1}) ($P < 0.01$) at same time. After 6 days treatment, both microorganisms, *S. aureus* and *P. aeruginosa*, were eradicated from all eyes of rabbits treated with microspheres. However, at the same time, SA alone formulation demonstrated about 50% decrease in bacterial colony counts for both *S. aureus* (1.27 ± 0.21 Log_{10} CFU mL^{-1}) ($P < 0.05$) and *P. aeruginosa* (1.50 ± 0.30 Log_{10} CFU mL^{-1}) ($P < 0.05$) compared to 3 days therapy, but could not eradicate the bacteria from rabbit eyes. Results obtained in this study proved that the SA-loaded bioadhesive microsphere formulation produced a significantly higher therapeutic efficacy compared to SA alone suspension and was perfectly tolerated without causing any ocular irritation as previously described by Safwat and Al-Kassas [51]. They showed that the bioadhesive Eudragit® microspheres increased the bioavailability and therapeutic effectiveness of gentamicin by prolonging its corneal retention and penetration. The use of microspheres accelerated

Table 3
Log number of corneal colony counts for groups infected with *P. aeruginosa* and *S. aureus* after 3 and 6 days treatment by SA-loaded polycarbophil microspheres (PO5).

Rabbit	3rd day*						6th day**					
	P. aeruginosa (Log_{10} CFU mL^{-1})			*S. aureus* (Log_{10} CFU mL^{-1})			*P. aeruginosa* (Log_{10} CFU mL^{-1})			*S. aureus* (Log_{10} CFU mL^{-1})		
	SA-loaded MS	SA alone	Negative control	SA-loaded MS	SA alone	Negative control	SA-loaded MS	SA alone	Negative control	SA-loaded MS	SA alone	Negative control
1	1.00	2.30	3.30	2.00	2.70	3.18	0.00	1.20	4.70	0.00	1.50	3.75
2	2.00	3.00	4.00	1.70	3.00	3.30	0.00	1.80	4.30	0.00	1.20	4.10
3	2.00	2.70	3.70	1.85	2.85	3.23	0.00	1.50	4.50	0.00	1.10	4.14
Average	1.66 ± 0.58	2.68 ± 0.35	3.67 ± 0.35	1.85 ± 0.15	2.85 ± 0.15	3.24 ± 0.06	0.00 ± 0.00	1.50 ± 0.30	4.50 ± 0.20	0.00 ± 0.00	1.27 ± 0.21	4.00 ± 0.21

* *P. aeruginosa*: $P_{Microsphere\ vs\ SA\ alone} < 0.05$; $P_{Microsphere\ vs\ Control} < 0.01$; $P_{SA\ alone\ vs\ Control} < 0.05$ *S. aureus*: $P_{Microsphere\ vs\ SA\ alone} < 0.001$; $P_{Microsphere\ vs\ Control} < 0.001$; $P_{SA\ alone\ vs\ Control} < 0.01$.
** *P. aeruginosa*: $P_{Microsphere\ vs\ SA\ alone} < 0.001$; $P_{Microsphere\ vs\ Control} < 0.001$; $P_{SA\ alone\ vs\ Control} < 0.001$ *S. aureus*: $P_{Microsphere\ vs\ SA\ alone} < 0.001$; $P_{Microsphere\ vs\ Control} < 0.001$; $P_{SA\ alone\ vs\ Control} < 0.001$.

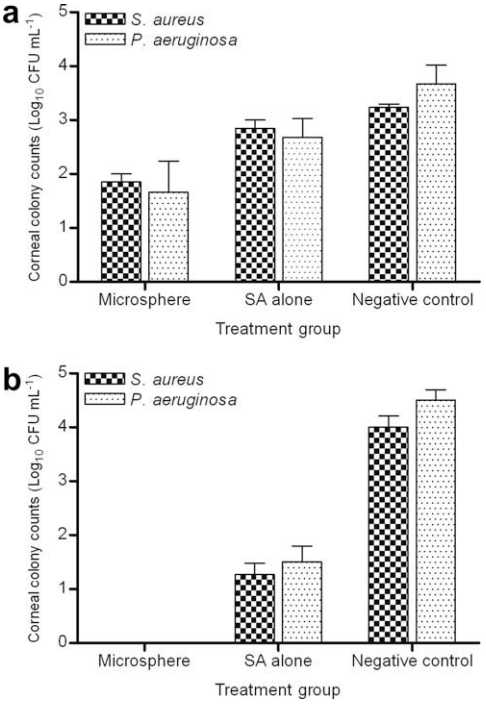

Fig. 4. Average corneal colony counts for groups infected with *P. aeruginosa* and *S. aureus* (a) after 3 and (b) after 6 days treatment.

Table 4
The clinical scores graded from absent to severe in groups treated with SA-loaded polycarbophil microspheres (PO5) and SA alone by number and percentage (a) after 3 days and (b) after 6 days treatment.

Evaluation	3rd day				6th day				$p^{1*}(X^2)$	$p^{2*}(X^2)$	$p^{1**}(X^2)$	$p^{2**}(X^2)$
	P. aeruginosa		*S. aureus*		*P. aeruginosa*		*S. aureus*					
	SA-loaded MS	SA alone	SA-loaded MS	SA alone	SA-loaded MS	SA alone	SA-loaded MS	SA alone				
Blepharitis												
0	4 (66.67)	0 (0.00)	4 (66.67)	0 (0.00)	3 (100.0)	1 (33.33)	2 (66.67)	0 (0.00)				
1	2 (33.33)	0 (0.00)	2 (33.33)	0 (0.00)	0 (0.00)	0 (0.00)	1 (33.33)	1 (33.33)	0.007	0.007	0.083	0.045
2	0 (0.00)	1 (16.67)	0 (0.00)	1 (16.67)	0 (0.00)	0 (0.00)	0 (0.00)	2 (66.67)	(12.00)	(12.00)	(3.00)	(4.00)
3	0 (0.00)	5 (83.33)	0 (0.00)	5 (83.33)	0 (0.00)	2 (66.67)	0 (0.00)	0 (0.00)				
Conjunctivitis												
0	3 (50.00)	0 (0.00)	3 (50.00)	0 (0.00)	2 (66.67)	0 (0.00)	2 (66.67)	0 (0.00)				
1	3 (50.00)	0 (0.00)	3 (50.00)	0 (0.00)	1 (33.33)	1 (33.33)	1 (33.33)	1 (33.33)	0.007	0.007	0.046	0.046
2	0 (0.00)	1 (16.67)	0 (0.00)	1 (16.67)	0 (0.00)	1 (33.33)	0 (0.00)	1 (33.33)	(12.00)	(12.00)	(3.89)	(3.89)
3	0 (0.00)	5 (83.33)	0 (0.00)	5 (83.33)	0 (0.00)	1 (33.33)	0 (0.00)	1 (33.33)				
Iritis												
0	3 (50.00)	0 (0.00)	3 (50.00)	0 (0.00)	2 (66.67)	0 (0.00)	3 (100.0)	0 (0.00)				
1	3 (50.00)	0 (0.00)	3 (50.00)	0 (0.00)	1 (33.33)	1 (33.33)	0 (0.00)	0 (0.00)	0.007	0.007	0.046	0.019
2	0 (0.00)	1 (16.67)	0 (0.00)	1 (16.67)	0 (0.00)	1 (33.33)	0 (0.00)	2 (66.67)	(12.00)	(12.00)	(3.89)	(5.55)
3	0 (0.00)	5 (83.33)	0 (0.00)	5 (83.33)	0 (0.00)	1 (33.33)	0 (0.00)	1 (33.33)				
Corneal oedema												
0	4 (66.67)	0 (0.00)	6 (100.00)	4 (66.67)	2 (66.67)	1 (33.33)	2 (66.67)	1 (33.33)				
1	0 (0.00)	2 (33.33)	0 (0.00)	2 (33.33)	1 (33.33)	0 (0.00)	1 (33.33)	1 (33.33)	0.046	0.188	0.023	0.251
2	0 (0.00)	2 (33.33)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	(8.00)	(3.34)	(5.35)	(1.32)
3	2 (33.33)	2 (33.33)	0 (0.00)	0 (0.00)	0 (0.00)	2 (66.67)	0 (0.00)	1 (33.33)				
Corneal infiltration												
0	4 (66.67)	0 (0.00)	5 (83.33)	0 (0.00)	3 (100.0)	0 (0.00)	3 (100.0)	0 (0.00)				
1	2 (33.33)	1 (16.67)	1 (16.67)	1 (16.67)	0 (0.00)	1 (33.33)	0 (0.00)	0 (0.00)	0.025	0.019	0.034	0.019
2	0 (0.00)	4 (66.67)	0 (0.00)	4 (66.67)	0 (0.00)	1 (33.33)	0 (0.00)	2 (66.67)	(9.33)	(10.00)	(4.50)	(5.55)
3	0 (0.00)	1 (16.67)	0 (0.00)	1 (16.67)	0 (0.00)	1 (33.33)	0 (0.00)	1 (33.33)				

Significances between SA-loaded microsphere formulation and SA alone for the group infected with *P. aeruginosa* (p^1) and group infected with *S. aureus* (p^2) after *3 days and **6 days treatment; X^2: Chi-square value; 0: absent; 1: mild; 2: moderate; 3: severe.

the healing rate and reduced the healing time when compared to commercial eye drops [51].

The clinical results obtained after 3 and 6 days of therapy were also parallel with results that belong to colony counts. Blepharitis, conjunctivitis, iritis, corneal oedema and corneal infiltration are the most important signs of bacterial keratitis. The results of the clinical examinations are presented in Table 4. Clinical presentation of eyes infected with *S. aureus* after treatment with microspheres is shown in Fig. 5. Microspheres (PO5) demonstrated significantly lower scores for all signs of infection compared with SA alone in both infection models after 3 and 6 days of treatment. The clinical scores and colony counts confirmed that therapy was successfully concluded with the treatment of SA-loaded polycarbophil microspheres.

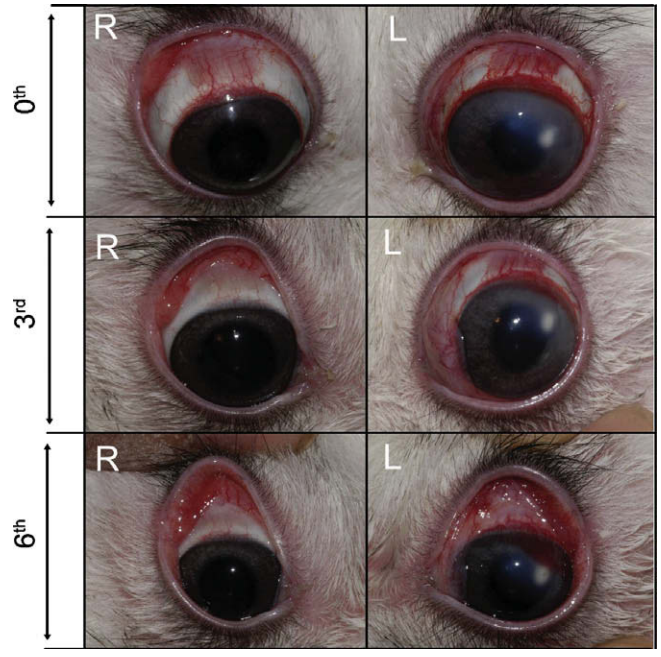

Fig. 5. Photographs demonstrate the typical clinical presentation of eyes infected with *S. aureus* after 3 and 6 days treatment with SA-loaded polycarbophil microspheres (right eyes, marked as R) and SA alone (left eyes, marked as L).

4. Conclusion

The *in vitro* and *in vivo* studies investigating the efficacy of SA-loaded microspheres in the treatment of the bacterial keratitis caused by *S. aureus* and *P. aeruginosa* demonstrated that the using of bioadhesive polymers to prepare microspheres enhanced therapeutic efficacy against both bacterial infection models. SA-loaded polycarbophil microsphere formulation completely eradicated the bacteria from eyes after 6 days of treatment, whereas SA alone suspension was found to be not clinically effective in the treatment of bacterial keratitis produced by *S. aureus* and *P. aeruginosa* at same time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2009.02.006.

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