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# Therapeutic efficacy of sustained drug release from chitosan gel on local inflammation

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**Abstract**

The model anti-inflammatory drug prednisolone (PS) was retained in chitosan (CS) gel beads, which were prepared in a 10% aqueous amino acid solution (pH 9.0). Sustained release of PS from the CS gel beads was observed. Carrageenan solution was injected into air pouches (AP), which were prepared subcutaneously on the dorsal surface of mice, in order to induce local inflammation. CS gel beads retaining PS were then implanted into the AP to investigate the therapeutic efficacy of sustained PS release against local inflammation. In vivo PS release from CS gel beads was governed by both diffusion of the drug and degradation of the gel matrix. Sustained drug release by CS gel beads allowed the supply of the minimum effective dose and facilitated prolonged periods of local drug presence. Inflammation indexes were significantly reduced after implantation of CS gel beads when compared with injection of PS suspension. Thus, extension of the duration of drug activity by CS gel beads resulted in improved therapeutic efficacy. These observations indicate that CS gel beads are a promising biocompatible and biodegradable vehicle for treatment of local inflammation.


*Keywords:* Chitosan; Sustained release; Inflammation; Biodegradation; Air pouch

## 1. Introduction

The prevalence of arthritis deformans (or rheumatoid arthritis) is currently increasing as society ages. Intra-articular injection of steroidal drugs is commonly used to treat this condition. However, achieving sustained intra-articular drug levels is difficult due to rapid drug clearance from the injection site, which is facilitated by drug dissolution. Under such conditions, patients are repeatedly subjected to intra-articular injection as needed, but higher intra-articular drug concentrations may harm articular cartilage and may also exert global effects after passing into the bloodstream. It is therefore necessary to control drug release using a vehicle. The search for adequate drug delivery systems has focused on attempts to design systems that improve the control of drug release. Although such systems would improve the efficacy of medical treatments, minimize side effects, and improve patient compliance, few studies have investigated the therapeutic efficacy of sustained drug release on local inflammations.

Materials that are implanted into the body must be non-toxic, biocompatible and biodegradable if they are to avoid the host's defense system for the duration of their contact with living structures. Chitosan (CS) is a

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plentiful natural polysaccharide that is non-toxic, biocompatible and biodegradable (Tomihata and Ikada, 1997; Onishi and Machida, 1999). Numerous studies have demonstrated that CS is an effective and safe vehicle for the sustained release of drugs (Yomota et al., 1990; Golomb et al., 1992; Jameela and Jayakrishnan, 1995; Oungbho and Müller, 1997). Preparation of CS microspheres has also been widely studied (Thanoo et al., 1992; Williams et al., 1998; He et al., 1999; Tokumitsu et al., 1999; Remuñan-López et al., 2000). Therefore, CS is promising as a material for implantable biomedical applications (Khor and Lim, 2003).

For drug delivery applications, CS gel is usually prepared by forming a Schiff base with glutaraldehyde, by forming an electeostatic complex with anionic polymers, via acylation with acetic anhydride, or under strong alkaline conditions. However, these conditions are not ideal due to the toxic nature of residual cross-linking reagents (Carreño-Gómez and Duncan, 1997). Drug decomposition due to strong alkalis or retention of organic solvents used to remove cross-linking reagents is also a concern. It is therefore desirable to prepare vehicles for drug delivery under milder condition. We have found that CS gel spheres form at around pH 9 in aqueous amino acid solution (Kofuji et al., 1999), which is much milder than the usual conditions (aqueous solutions of pH > 12). Drug release from gel beads prepared at pH 9 was reduced when compared with that from beads prepared at pH > 12. This is because the molecular weight of CS did not decrease after gel preparation at the lower pH (Kofuji et al., 2003). In vitro and in vivo drug release as well as the biodegradation properties of CS gel beads depend on CS species, particularly with regard to molecular weight and degree of deacetylation, and on the formation of a complex between chondroitin sulfate (Cho) and CS (Kofuji et al., 2000, 2001, 2002).

It has also been reported that CS acts on the epiphyseal cartilage and is able to augment wound healing in the articular cartilage (Lu et al., 1999; Suh and Matthew, 2000; Lahiji et al., 2000). Inhibition of pro-inflammatory cytokine production (Kim et al., 2002), and analgesic effects of chitosan on inflammatory pain (Okamoto et al., 2002) have also been demonstrated. Furthermore, because Cho, a biocompatible and biodegradable mucopolysaccharide that is similar to CS, is a component of cartilage and is clinically used in healing of articular cartilage, CS and Cho-modified CS gel beads may be able to assist in wound healing after fulfilling their role in drug delivery.

In the present study, we investigated the therapeutic efficacy of sustained drug release by CS gel beads implanted into subcutaneous air pouches (AP) prepared on the dorsal surfaces of mice, in which local inflammation was induced by carrageenan (CA) injection and observed the profiles of in vivo drug release from the CS gel beads.

## 2. Materials and methods

### 2.1. Materials

CS was purchased from Katokichi Co. Ltd. (Japan). CA, peroxidase from horseradish (100 U/mg), and lactic dehydrogenase (LDH) activity determination kit (LDH-UV test, Wako) were purchased from Wako Pure Chemical Industries (Japan). Prednisolone (PS) was purchased from Nacalai Tesque Inc. (Japan). BCA protein assay reagent was purchased from Pierce Chemical Co. (USA). All other chemicals were of reagent grade.

### 2.2. Determination of degree of CS deacetylation

Degree of CS deacetylation was determined by colloidal titration. CS (0.5%, w/w) was dissolved in aqueous acetic acid solution (5%, v/w). One gram of CS solution was added in flask, and was diluted to 30 ml with distilled/demineralized water. After adding 100 μl of 0.1% (w/v) toluidine blue indicator solution, the sample solution was titrated against 1/400N potassium polyvinyl sulfate solution. A single molecule of potassium polyvinyl sulfate reacts with each deacetylated amino group in the CS molecule. The degree of deacetylation was then calculated from the molar concentration of potassium polyvinyl sulfate solution consumed.

### 2.3. Molecular weight determination of CS

CS was subjected to gel permeation chromatography (GPC). CS (0.1%, w/w) was dissolved in 0.1 M acetate buffer (pH 4.5). A 20-μl aliquot of the sample

was loaded onto a column for GPC (Shodex SB-806M HQ; 300 mm × 8.0 mm) and was eluted with 0.1 M acetate buffer (pH 4.5) as the mobile phase at a flow rate of 0.5 ml/min (Shimadzu LC-10AS). CS in the effluent was detected with a refractive index detector (Shimadzu RID-10A). Molecular weight standards were eight types of pullulan with molecular weights ranging between 5900 and 788,000 (Shodex STANDARD P-82). Molecular weight was estimated from a calibration curve produced using these standards. Viscosity of CS solution was also determined at 37 °C using a B type viscometer (Tokyokeiki).

### 2.4. Preparation of CS gel beads for dissolution test

CS gel beads were prepared as follows. CS (1%, w/w) was dissolved in 0.1 M acetate buffer (pH 4.5) and 15% (w/w) PS was then added to the CS solution. Ten drops (about 0.56 g) of this suspension, theoretically containing 84 mg of PS, was slowly added dropwise into 20 ml of 10% (w/v) aqueous glycine solution (pH 9.0) using a pipette and left to stand at room temperature for 25 min. CS hydrogel beads formed spontaneously and were dried at 37 °C for 24 h in a dish before desiccating under vacuum in the presence of $P_2O_5$. The amount of PS in the gel beads was calculated by subtracting the amount of PS detected in the preparation solution after bead formation from the theoretical total amount of PS added to the initial CS solution. The dried CS gel beads retained more than 99.5% of the theoretical total amount of PS, and had a diameter of $5.9 \pm 0.9$ mm.

### 2.5. Preparation of CS gel beads for implantation

CS gel beads for implantation were prepared a single bead at a time, as described above. Before implantation the dried beads were washed with distilled/demineralized water and returned to the hydrogel in order to remove glycine, which may adversely affect living tissue, and had a diameter of $5.8 \pm 1.3$ mm. A single bead retained about $8.3 \pm 0.2$ mg of PS.

### 2.6. Dissolution test

The rate of PS release from CS gel beads into 0.1 M phosphate buffer (pH 7.2) was determined. Dried gel beads were added to 500 ml of dissolution medium in a Japanese Pharmacopoeia Thirteenth Edition (JP XIII) dissolution test apparatus (paddle method, 100 rpm, 37 °C). A 4-ml aliquot of the solution was periodically removed for analysis and replaced with 4 ml of the dissolution medium (pre-warmed to 37 °C) in order to maintain a constant volume. The absorbance of the test solution was determined with a spectrophotometer (Shimadzu UV-1200) at 246 nm. The amount of PS released from CS gel beads was estimated from a standard curve produced in advance. All dissolution tests were performed in triplicate.

### 2.7. Animal studies

Mice having induced local inflammation were prepared as follows. Air (3 ml) was injected subcutaneously into the dorsal surface of mice (ddy, male, aged 5 weeks) to form air pouches. An oval AP was formed after an additional 1 ml of air was injected at days 1 and 4. CA (30 mg/ml), which is known to induce inflammation (Hambleton and Miller, 1989), was dissolved in warm physiological saline, and 1 ml of this was injected into the AP. Under ether anesthesia, an incision (about 1 cm) was made in the AP, and a single bead retaining PS ($8.3 \pm 0.2$ mg) was implanted immediately after injection of CA solution. In addition, 10% PS was suspended in physiological saline and 83 μl of this suspension (i.e. 8.3 mg as PS) was injected with mock implantation by the same method. As controls, physiological saline (1083 μl; same volume as under experimental conditions) was injected with mock implantation into AP, and CS gel beads without PS were also implanted as described above. After a predetermined period of time, mice were injected with 0.7% (w/v) Evans blue solution dissolved in physiological saline (10 μl/g body weight) intravenously into the tail vein in order to investigate the vascular permeability of Evans blue. The abdominal skin was then opened under anesthesia with pentobarbital sodium and blood was collected from the vena cava caudalis 30 min after injection of Evans blue solution. Following dislocation of the cervical vertebrae, beads were retrieved from the AP, and AP exudates were collected and weighed. Contents of the AP were then collected by washing with 1 ml of physiological saline three times, and the dorsal surface skin forming the AP was collected and weighed. Mice used for determination of MPO activity, LDH activity, and

PS concentration in serum and AP contents were not injected with Evans blue solution in order to minimize interference with accurate determination. The following indicators were determined for evaluation of therapeutic efficacy on local inflammation: amount of exudate in AP; amount of protein in AP contents; amount of Evans blue in AP contents and in serum; myeloperoxidase (MPO) activity; LDH activity in blood, AP contents and per defined area of dorsal surface skin forming the AP; and histological examination. PS concentrations in the serum and amount of PS in the gel beads and in AP contents were also determined. All research protocols were approved by the Committee for Animal Research at Hokuriku University.

### *2.8. Determination of protein in AP contents*

Contents collected from the AP were made up to 5 ml with physiological saline. The amount of protein in the AP contents was determined by the BCA method using protein assay reagent. Absorbance of the BCA test solution was determined with a multi-spectrophotometer (Dainippon Viento) at 562 nm. The amount of protein was estimated from a standard curve produced using bovine serum albumin.

### *2.9. Vascular permeability determination of Evans blue*

Concentration of Evans blue was determined as follows. Serum (50 μl) was made up to 5 ml with physiological saline. AP contents were also made up to 5 ml with physiological saline. Samples of AP contents containing blood were excluded. Absorbance of each sample was determined using a multi-spectrophotometer (Dainippon Viento) at 606 nm. Concentration of Evans blue was estimated from a standard curve produced in advance. Vascular permeability was assessed based on the ratio between the amount of Evans blue in AP contents to its concentration (mg/ml) in serum.

### *2.10. LDH activity determination*

LDH activity in blood, in the AP contents and in a defined area of the dorsal surface skin forming the AP (diameter was 9 mm) was determined as follows. Samples without injection of Evans blue were used in this determination. Blood was centrifuged at 3500 rpm for 5 min, and the supernatant was collected. AP contents were made up to 5 ml with physiological saline before use. Skin samples were homogenized in twenty times the skin weight of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide to dissolve the enzyme. Homogenized skin samples were repeated freezing and thawing three times to ensure cell destruction. Freezed samples were fused at room temperature, and were centrifuged at 3500 rpm for 15 min. The supernatant of homogenized skin samples was then used. LDH activity was determined using the Wróblewski-La Due method using an LDH determination kit (LDH-UV Test, Wako, Wako Pure Chemical Industries, Japan). Fifteen milliliters of 0.18 mM reduced β-nicotinamide adenine dinucleotide (NADH) solution and 15 ml of 0.62 mM lithium pyruvate in 50 mM phosphate buffer (pH 7.5) were mixed, and warmed at 35 °C. The samples (0.05 ml) were then added to 2 ml of this mixture. Absorbance of the test solution at 2 and 4 min after addition of sample was determined with a spectrophotometer (Shimadzu UV-1200) at 340 nm. The difference in absorbance at 2 and 4 min was converted to LDH activity using the table supplied with the kit. LDH activity (IU/l) was thus obtained as the product of the difference in absorbance and a coefficient (1720).

### *2.11. MPO activity determination*

MPO activity in blood, in AP contents and in a defined area of the dorsal surface skin forming the AP (diameter was 9 mm) was determined using the same samples as for LDH activity determination, as described above. A total of 0.3 ml of 2 mg/ml *o*-dianisidine hydrochloride as a color former, 1.5 ml of 50 mM phosphate buffer (pH 6.0), and 0.6 ml distilled/demineralized water were mixed, and warmed at 25 °C. Just prior to measurement, 0.3 ml of 0.006% (v/v) hydrogen peroxide solution was added to the mixture, and then 0.3 ml of the sample was added. Absorbance of the test solution at 30 and 90 s after addition of sample was determined using a spectrophotometer (Shimadzu UV-1200) at 460 nm. The difference in absorbance at 30 and 90 s was regarded as MPO activity per defined area (diameter was

9 mm) of the dorsal surface skin forming the AP (IU) or the activity in AP contents (IU/ml), as estimated from a standard curve produced simultaneously using peroxidase.

### 2.12. Histopathological examination

Tissue collected from the dorsal surface skin forming the AP was fixed in a solution of 4% (w/v) paraformaldehyde containing 1% (v/v) glutaraldehyde, and fixed tissues were then neutralized by 10 mM phosphate buffer (pH 7.5), and water and fat were removed using a graded series of ethylalcohol (50–100%, v/v). Treated tissue was embedded in hydroxypropyl methyl metacrylate containing 10% (v/v) water-soluble methacrylate and 0.1% (w/v) M type water-soluble methacrylate and 25% (v/v) methyl metacrylate, and was incubated at 65–70 °C for 20 h. Embedded tissue was sectioned by microtome. Sections were stained with hematoxylin and eosin, and were observed under a microscope (Nikon ECLIPSE E600W).

### 2.13. Determination of PS in serum and AP contents in mice with inflammation

Blood samples were centrifuged at 3500 rpm for 5 min. AP contents were made up to 5 ml with physiological saline before use. When PS in the AP contents was not completely dissolved, AP contents were diluted with physiological saline. Blood supernatants or AP content samples (100 μl) were then added to 500 μl of 20% trichloroacetic acid aqueous solution to remove proteins, 200 μl of 4 μg/ml aqueous methyl *p*-hydroxybenzoate solution as an internal standard, and 200 μl of distilled/demineralized water. The mixture was centrifuged at 3500 rpm for 5 min and the supernatant was loaded into a sample extraction product (OASIS HLB, Waters). The extracted sample was subjected to high-performance liquid chromatography (HPLC). A 50-μl aliquot of the sample was loaded onto a column (SHISEIDO CAPCELL PAK C18 UG120; 100 mm × 4.6 mm) and was eluted with 25% acetonitrile/aqueous acetate buffer solution (pH 4.5) as the mobile phase at a flow rate of 0.3 ml/min (Shimadzu LC-10AS). PS in the effluent was detected at 246 nm using a UV spectrophotometer (Shimadzu SPD-10AVVP).

### 2.14. Determination of PS in AP in mice without inflammation

AP contents were made up to 5 ml with physiological saline before. When PS in the AP contents was not completely dissolved, AP contents were diluted with physiological saline. This solution (900 μl) was added to 100 μl of 20 μg/ml of internal standard in 25% acetonitrile/aqueous acetate buffer solution (pH 4.5). This mixture was centrifuged at 3500 rpm for 5 min, and the supernatant was filtered (Cosmonice Filter S, 0.5 μm, Millipore) and subjected to HPLC as described above.

### 2.15. Determination of PS in gel beads

CS gel beads were dissolved in 2 ml of 0.1 M acetate buffer (pH 4.5). These samples were made up to 20 ml with 25% acetonitrile/aqueous acetate buffer solution (pH 4.5). This solution (900 μl) was added to 100 μl of 20 μg/ml internal standard in 25% acetonitrile/aqueous acetate buffer solution (pH 4.5). The mixture was centrifuged at 3500 rpm for 5 min, and the supernatant was filtered (Cosmonice Filter S, 0.5 μm, Millipore) and subjected to HPLC as described above.

### 2.16. Statistical analysis

Data are represented as mean ± S.D. and were analyzed statistically using Student's *t*-test after *F*-test or Smirnov–Grubbs' outlier test ($\alpha = 0.05$).

## 3. Results and discussion

### 3.1. CS properties and gel bead formation

CS with a weight-average molecular weight of 980,000 Da and 91% deacetylation was used in this study. Spontaneous formation of spherical CS gel beads was observed due to coacervation when 1% (w/w) CS solution, which was dissolved in 0.1 M acetate buffer (pH 4.5), containing 15% (w/w) of PS was placed into 10% (w/v) aqueous glycine solution (pH 9.0).

In a previous study, four CS species, ranging from 160,000 to 2,800,000 Da in weight-average molecular weight and from 70 to 100% in deacetylation, were investigated. The ease of CS gel bead preparation was

observed to depend on various CS properties, such as molecular weight and degree of deacetylation. Dissolution of CS was aided by acetic acid, which triggers salt formation with some amino groups of CS. When CS solution containing acetic acid was added dropwise into 10% aqueous amino acid solution (pH 9.0), CS-acetic acid salts dissociated more rapidly in the solution than in the aqueous solution without amino acid, and spherical gel formation was observed. It is important that bead formation occurs rapidly after CS is added to the preparative medium because this enables more efficient drug encapsulation by inhibiting escape of the drug into the preparative medium. High concentrations of CS species with high molecular weights could not be used to prepare CS gel beads due to their high viscosity in solution, which prevented them from being dropped by pipette into the preparative medium. The use of very low concentrations of CS did not result in instantaneous bead formation because the diffusion rate of CS within the preparative medium was too rapid. Thus, instantaneous formation of spherical gel particles is achieved because a sufficient number of CS amino groups are sufficiently close to one another to allow for bead formation. Therefore, CS concentration must be sufficiently high in order to prevent rapid diffusion of CS amino groups away from one another. On the other hand, CS with 100% of deacetylation (weight-average molecular weight: 160,000) did not form gel spheres in the present preparative medium, even at higher concentrations. Furthermore, addition of higher concentrations of drug to the CS solution raised the viscosity of the solution.

The biodegradation of CS gel beads is also governed by the properties of CS, particularly the degree of deacetylation of CS, and was accelerated as the degree of deacetylation decreased. CS gel beads with lower degrees of deacetylation (i.e. 70 and 82%) degraded within 3 days after implantation into subcutaneous AP prepared on the dorsal surface of mice. On the other hand, CS gel beads with 91% deacetylation which were used in this experiment remained even after 14 days in the AP of mice. It was necessary for the CS gel beads to remain in the implantation site for the duration of the experimental period. Therefore, an optimum concentration (1%) of CS with 91% deacetylation, which maintained a spherical shape when CS was placed into 10% aqueous glycine solution (pH 9.0), was employed in this study. These CS gel beads retained more than 99.5% of the theoretical total amount of PS.

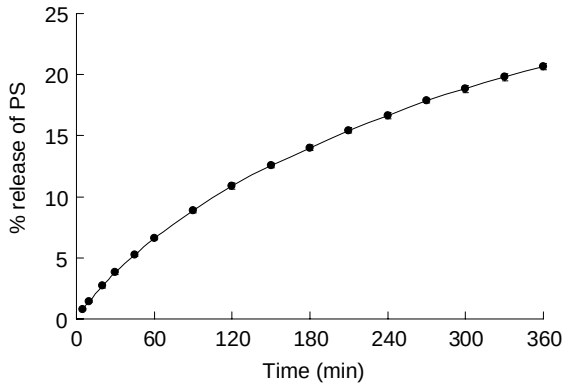


Fig. 1. In vitro release profiles of PS from CS gel beads. (●): 1% CS gel beads retaining PS (about 83.6 mg) prepared in 10% aqueous glycine solution (pH 9.0). Dissolution test: JP XIII dissolution test apparatus (paddle method, 100 rpm), 500 ml of 0.1 M phosphate buffer (pH 7.2), 37 °C. Data represent mean ± S.D. ($n = 3$).

### 3.2. In vitro release of PS from CS gel beads

PS powder dissolved immediately in the dissolution medium. In contrast, as shown in Fig. 1, sustained release of PS from CS gel beads was observed; only about 20% of PS retained in CS gel beads was released after 6 h. In addition, the CS gel beads maintained their shape over time. In vitro release of PS from CS gel beads would thus be controlled by diffusion of PS from the gel matrix.

### 3.3. Induction of inflammation

Local inflammation in mice was induced by injection of CA solution into AP prepared on the dorsal surface. As inflammation progresses, telangiectatic vessels are generally induced, and the ingredients in serum are exuded to the outside from blood vessels as the vascular permeability increases. Aggressive migration of inflammatory cells, such as leukocytes, then occurs. Therefore, initially, the amount of exudate in the AP, the amount of protein in the AP contents, and the vascular permeability of Evans blue were determined in order to confirm the induction of inflammation by CA. As shown in Fig. 2a, the amount of exudate in AP

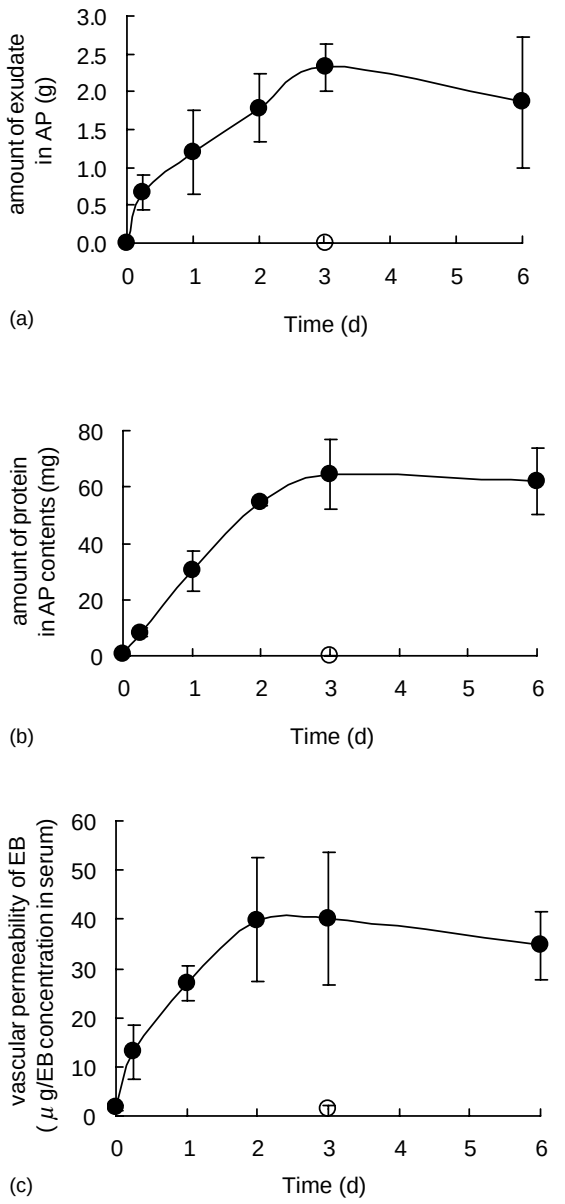


Fig. 2. Profiles of various inflammation indicators after injection of CA solution or physiological saline with mock implantation: (a) amount of exudate in AP ($n = 6$–17), (b) amount of protein in AP contents ($n = 6$–16), (c) vascular permeability of Evans blue (EB). Vascular permeability was assessed based on the ratio between the amount of EB in AP contents and its concentration (mg/ml) in serum ($n = 4$–7). (●): 3% CA (1 ml) dissolved in physiological saline, (○): physiological saline (1.083 ml). Data represent mean ± S.D. Data were analyzed statistically using Student's *t*-test after *F*-test and Smirnov–Grubbs' outlier test ($\alpha = 0.05$).

increased gradually, and reached a maximum value at 3 days after injection of CA solution, and this value remained constant. Similar results were obtained for the amount of protein amount in the AP contents after injection of CA solution (Fig. 2b). The degree of vascular permeation of Evans blue rose gradually and reached a maximum value at 2 days after injection of CA solution (Fig. 2c), and this value remained constant. These findings suggest that inflammation was induced by injection of CA, and that these indicators of inflammation corresponded well with the stage of inflammation.

In contrast, no increase of any of the indicators was observed after injection of physiological saline alone accompanied by mock implantation. This indicates that neither injection of physiological saline nor the incision of AP during implantation of CS gel beads influenced any of the inflammation signs. Therefore, increases in these indicators were the result of inflammation, and were not related to injection volume or incision of AP. Within 3 days of CA injection, signs of inflammation in the AP were clearly seen and bleeding was observed in some mice at 6 days after injection. Furthermore, ulcers were observed after 14 days. As a result, therapeutic efficacy of CS gel beads retaining PS, which were implanted immediately after CA solution was injected, was evaluated at 3 days after CA injection in all subsequent studies.

### *3.4. Therapeutic efficacy of CS gel beads retaining PS*

Steroidal anti-inflammatory drugs are known to inhibit inflammation by blocking phospholipase $A_2$ activity, thus inhibiting production of chemical mediators, such as prostaglandins and leukotrienes. Furthermore, these drugs inhibit the production of various cytokines and this also inhibits inflammation. Thus, steroidal drugs inhibit telangiectasia, the increase in vascular permeability, and the infiltration of inflammatory cells. The following indicators were therefore measured in order to evaluate the therapeutic efficacy of sustained release of a steroidal drug (PS) from CS gel beads and to compare this with the efficacy of drug injection: (1) amount of exudate in AP, (2) amount of protein in AP contents, (3) vascular permeability of Evans blue, (4) LDH activity in AP contents, (5) MPO activity in AP contents and in a

defined area of the dorsal surface skin forming the AP, and (6) histological examination. As controls, these indicators were also measured in mice injected with physiological saline alone and those implanted with CS gel beads without PS. The weight of dorsal surface skin forming the AP per defined area (diameter was 9 mm), LDH activity in a defined area of the dorsal surface skin forming the AP and in serum, and MPO activity in serum were also determined, but significant differences were not observed between the normal and inflamed values. Therefore, these values were not used for analytical purposes in the present study.

### 3.4.1. Amount of exudate in AP

The amount of exudate in AP increased gradually after injection of CA solution. Values after injection of PS suspension or implantation of CS gel beads retaining PS were similar to those after CA injection only until 1 day post-injection. However, the values after injection of PS suspension remained at the 1-day levels and never exceeded those observed after injection of CA alone, and at 3 days after injection, the values for PS suspension were significantly lower than those for CA injection alone. In contrast, values after implantation of CS gel beads retaining PS decreased gradually from 1 day, and values at 3 days after implantation were lower than those after injection of PS suspension. In fact, at 3 days values were at the same level as those for saline injection, which exhibited no exudates in AP after 3 days (Fig. 3a). On the other hand, CS gel beads without PS had no effect on values, which were the same as with injection of CA alone. These results indicate that injection of PS suspension decreased the amount of exudates in AP. Moreover, sustained release of PS by CS gel beads was more effective than PS alone, but CS alone had no reductive effect on exudate in AP.

### 3.4.2. Amount of protein in AP contents

The amount of protein in the AP is indicative of progress of the inflammatory response. Similar results were obtained for the amount of protein in AP contents as were observed for exudate in AP. The amount of protein in AP contents increased gradually after injection of CA solution. However, values after injection of PS suspension or implantation of CS gel beads retaining PS were significantly lower than those after CA injection alone at 1 day post-injection. At that

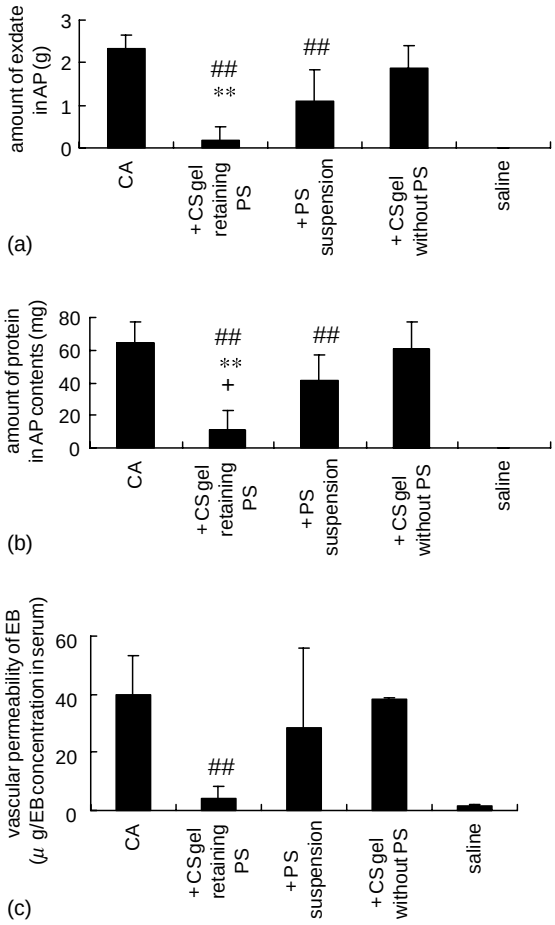


Fig. 3. Evaluation of therapeutic efficacy of CS gel beads retaining PS on various inflammation indicators at 3 days after implantation and injection of CA solution: (a) amount of exudate in AP ($n = 6$–12), (b) amount of proteins in AP contents ($n = 7$–12), (c) vascular permeability of Evans blue (EB). The vascular permeability was assessed based on the ratio between the amount of EB in AP contents to its concentration (mg/ml) in serum ($n = 3$–9). CA: injection of only 3% CA solution (1 ml), +CS gel retaining PS: implantation of CS gel beads retaining PS (8.3 mg) after injection of CA solution, +PS suspension: injection of PS (8.3 mg) suspension with mock implantation after injection of CA solution, +CS gel without PS: implantation of CS gel beads without PS after injection of CA solution, saline: injection of physiological saline (1.083 ml) with mock implantation without injection of CA solution. Data represent mean ± S.D. Data were analyzed statistically using Student's *t*-test after *F*-test and Smirnov–Grubbs' outlier test ($\alpha = 0.05$). $^{\#\#} P < 0.01$: significantly different from CA, $^{**} P < 0.01$: significantly different from PS suspension, $^{+} P < 0.05$: significantly different from saline.

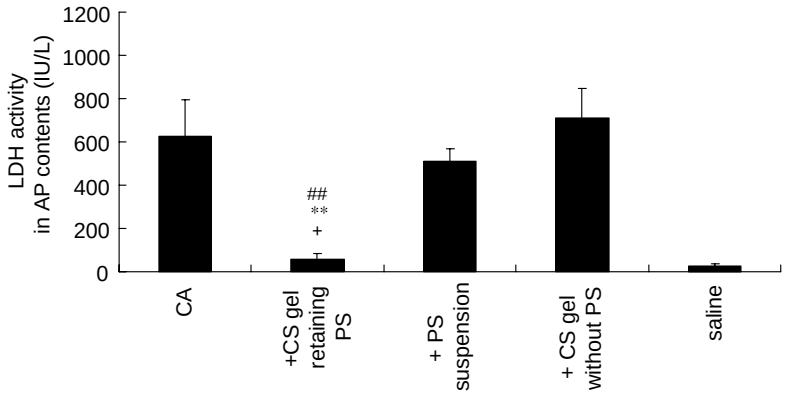


Fig. 4. Evaluation of therapeutic efficacy of CS gel beads retaining PS on LDH activity at 3 days after implantation and injection of CA solution. CA: injection of only 3% CA solution (1 ml), +CS gel retaining PS: implantation of CS gel beads retaining PS (8.3 mg) after injection of CA solution, +PS suspension: injection of PS (8.3 mg) suspension with mock implantation after injection of CA solution, +CS gel without PS: implantation of CS gel beads without PS after injection of CA solution, saline: injection of physiological saline (1.083 ml) with mock implantation without injection of CA solution. Data represent mean ± S.D. ($n = 4$–6). Data were analyzed statistically using Student's *t*-test after *F*-test and Smirnov–Grubbs' outlier test ($\alpha = 0.05$). $^{\#\#}P < 0.01$: significantly different from CA, $^{**}P < 0.01$: significantly different from PS suspension, $^{+}P < 0.05$: significantly different from saline.

time, similar values were seen for injection of PS suspension and implantation of CS gel beads retaining PS. After that, values for injection of PS suspension increased gradually, while values for implantation of CS gel beads retaining PS continued to decrease. As shown in Fig. 3b, values at 3 days after implantation of CS gel beads retaining PS were significantly lower than those for injection of PS suspension, but these values were not as low as those seen for saline injection alone.

### 3.4.3. Vascular permeability of Evans blue

Vascular permeability of Evans blue, which is commonly used to assess vascular permeability induced by inflammation because Evans blue binds completely with plasma proteins, was evaluated. Fig. 3c shows the values at 3 days after injection. Implantation of CS gel beads retaining PS tended to inhibit vascular permeability of Evans blue when compared with injection of PS suspension, but a significant difference was not observed, although a larger deviation was obtained with injection of PS suspension. However, a significant difference was not observed between implantation of CS gel beads retaining PS and injection of saline, which indicates that CS gel beads retaining PS inhibited vascular permeability to the levels observed for injection of saline.

### 3.4.4. LDH activity in AP contents

The extent of LDH activity reflects the extent of tissue destruction. Values after injection of PS suspension and implantation of CS gel beads retaining PS with CA injection were similarly increased until 1 day post-injection. However, the values for injection of PS suspension rose gradually after 1 day, while those for implantation of CS gel beads retaining PS were reduced. As shown in Fig. 4, the values after 3 days were not significantly reduced, even after injection of PS suspension, but values after implantation of CS gel beads retaining PS were significantly lower than those after both injection of CA or PS suspension. However, these values were not as low as those observed after saline injection.

### 3.4.5. MPO activity in a defined area of the dorsal surface skin forming the AP and AP contents

MPO is a type of hemoprotein that is present in the lysosomes and azurophil granules of neutrophil leucocytes, monocytes, and macrophages. MPO activity is known to be proportional to the quantity of these inflammatory cells induced by inflammation. MPO activity in a defined area of the dorsal surface skin forming the AP after injection of PS suspension increased for 5 h, and remained at this level for 1 day, and further increased after 1 day. In contrast, after

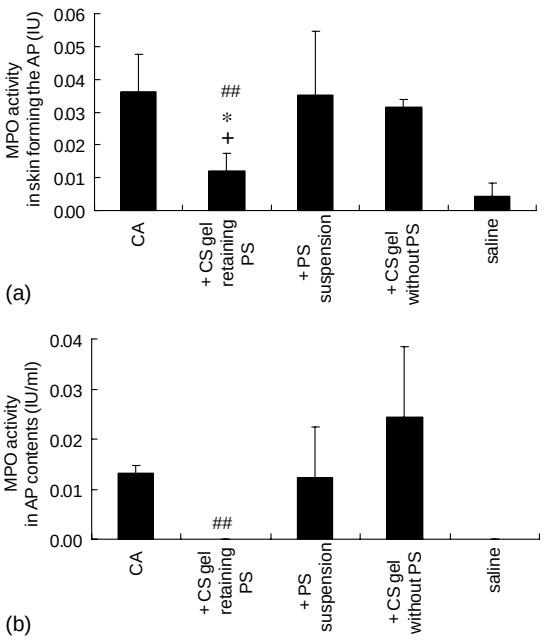


Fig. 5. Evaluation of therapeutic efficacy of CS gel beads retaining PS on MPO activity at 3 days after implantation and injection of CA solution: (a) MPO activity in a defined area (diameter was 9 mm) of the dorsal surface skin forming the AP ($n = 4$–6), (b) MPO activity in AP contents ($n = 5$). CA: injection of only 3% CA solution (1 ml), +CS gel retaining PS: implantation of CS gel beads retaining PS (8.3 mg) after injection of CA solution, +PS suspension: injection of PS (8.3 mg) suspension with mock implantation after injection of CA solution, +CS gel without PS: implantation of CS gel beads without PS after injection of CA solution, saline: injection of physiological saline (1.083 ml) with mock implantation without injection of CA solution. Data represent mean ± S.D. Data were analyzed statistically using Student's *t*-test after *F*-test and Smirnov–Grubbs' outlier test ($\alpha = 0.05$). $^{\#\#}P < 0.01$: significantly different from CA, $^{*}P < 0.05$: significantly different from PS suspension, $^{+}P < 0.05$: significantly different from saline.

implantation of CS gel beads, these values increased gradually within 5 h, but no further increase was observed. The values at 3 days after implantation of CS gel beads were significantly lower than those after injection of PS suspension, but were not as low as the levels observed after saline injection (Fig. 5a). On the other hand, MPO activity in AP contents after implantation of CS gel beads retaining PS was similar to that observed after saline injection, but values at 1 day after implantation tended to be higher than those after saline injection. MPO activity in AP contents at 3 days after implantation of CS gel beads retaining PS was not significantly lower than that observed for PS suspension due to the larger deviation observed with the PS suspension. However, MPO activity was reduced to the levels seen after injection of saline (Fig. 5b). On the other hand, MPO activity from 1 to 5 h after injection of PS suspension could not be determined because the comparatively higher concentration of PS hindered the determination of MPO activity.

### 3.4.6. Histological examination

The tissue sections that formed the AP were histologically observed by microscope (Nikon ECLIPSE E600W) in order to confirm the results described above regarding the therapeutic efficacy of sustained release of PS by CS gel beads. The number of inflammatory cells, such as leukocytes, lymphoid cell and monocytes, stained with hematoxylin and eosin were also counted. Infiltration of inflammatory cells was observed immediately after injection of CA solution into the AP. The average of number of inflammatory cells per unit area (1 $mm^2$) exceeded 350 cells after 6 h, and this value remained constant until 3 days post-injection. The number of inflammatory cells increased after 3 days, exceeding 600 cells at 6 days. Fig. 6 shows the number of inflammatory cells at 3 days after implantation. The values at 3 days after implantation of CS gel beads tended to be lower than those after injection of CA solution or PS suspension, but a significant difference was not observed. On the other hand, infiltration of inflammatory cells was also observed after incision without injection of CA solution, and the number of inflammatory cells following incision only was similar to that observed after implantation of CS gel beads. The extent of inflammation at this stage did not generally agree with the number of inflammatory cells. Therefore, the number of inflammatory cells may not accurately indicate the extent of inflammation, and thus detailed histological observation was also carried out.

Fig. 7 shows representative micrographs of histological sections of skin at 3 days after implantation. In the groups injected with CA solution, substantial infiltration inflammatory cells was observed, and numerous edemas of epithelial cells and interstitial tissue were also recognized, when compared to the groups receiving no injection or saline injection. On the other hand, after injection of PS suspension or implantation

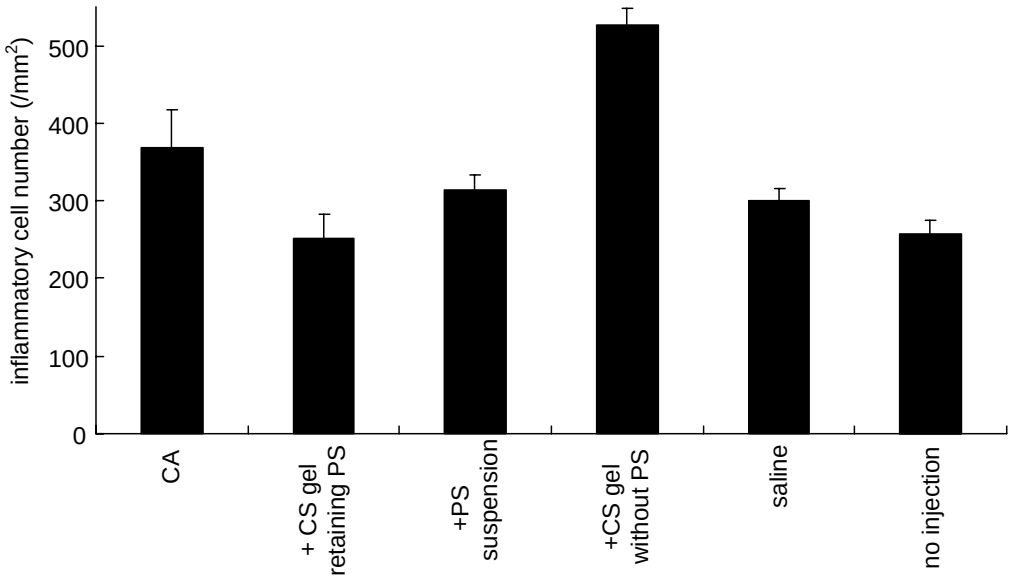


Fig. 6. Evaluation of therapeutic efficacy of CS gel beads retaining PS on number of inflammatory cells at 3 days after implantation and injection of CA solution. CA: injection of only 3% CA solution (1 ml), +CS gel retaining PS: implantation of CS gel beads retaining PS (8.3 mg) after injection of CA solution, +PS suspension: injection of PS (8.3 mg) suspension with mock implantation after injection of CA solution, +CS gel without PS: implantation of CS gel beads without PS after injection of CA solution, saline: injection of physiological saline (1.083 ml) with mock implantation without injection of CA solution, no injection: mock implantation only. Data represent mean ± S.D. ($n = 4$–10). Data were analyzed statistically using Student's $t$-test after $F$-test and Smirnov–Grubbs' outlier test ($\alpha = 0.05$).

of CS gel beads retaining PS, the number of inflammatory cells was smaller than that after injection with CA alone or implantation of CS gel beads without PS. Edemas of epithelial cells and interstitial tissue were also improved. These histological observations were consistent with the results for the various indicators of inflammation described above.

Taken together, the above results indicate that implantation of CS gel beads retaining PS exert improved therapeutic efficacy with regard to all indicators of inflammation.

### *3.5. PS behavior after implantation of CS gel beads retaining PS*

The in vivo release of PS from gel beads was evaluated by measuring the PS concentrations in serum, the amount of PS in the AP contents, and the percentage of residual PS in the beads and AP compared with the amount administered after implantation of CS gel beads retaining PS or injection of PS suspension.

As shown Fig. 8, when PS (8.3 mg) suspended in physiological saline was injected into the AP, it immediately started disappearing from the site, dissolving in the exudate and entering the bloodstream, and did not remain in the AP for long. In contrast, near-constant levels of PS in the AP were maintained after implantation of CS gel beads retaining PS. This indicates that PS was released into the AP more gradually from the CS gel beads, thus prolonging the duration of drug activity.

As shown in Fig. 9, serum levels changed gradually according to the amount of residual PS in the AP as it passed rapidly into the bloodstream. Comparatively higher serum concentrations of PS were observed immediately after injection of PS suspension, and average serum concentrations of PS reached 2.9 μg/ml after 1 h, with the maximum concentration of 3.4 μg/ml ($C_{max}$) being observed after 3 h, and PS was not observed in serum after 1 day. After implantation of CS gel beads retaining PS, lower serum concentrations, or even concentrations below the detection limit (0.1 μg/ml), were consistently seen.

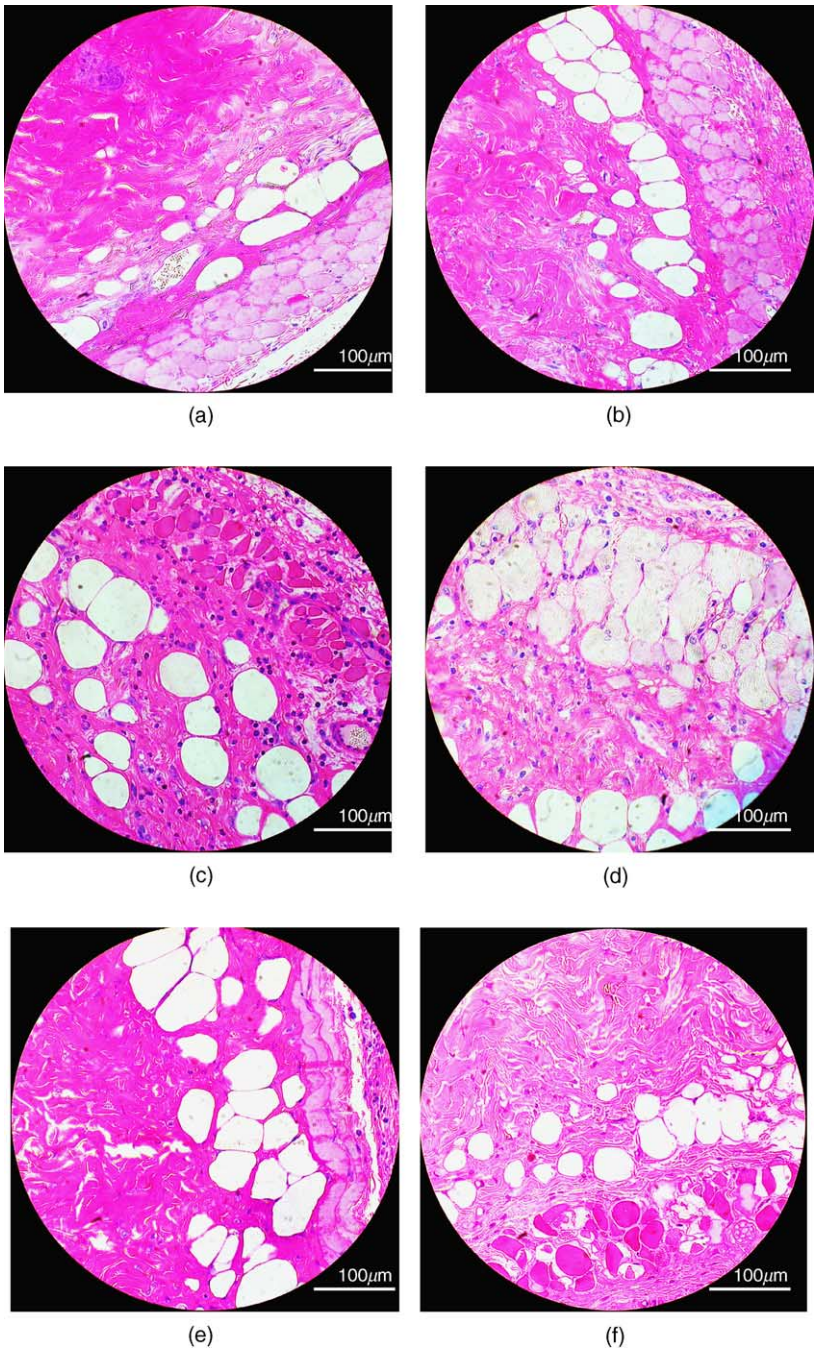


Fig. 7. Typical micrographs of histological sections at 3 days after implantation and injection of CA solution: (a) no injection: mock implantation only, (b) saline: injection of physiological saline (1.083 ml) with mock implantation without injection of CA solution, (c) CA: injection of only 3% CA solution (1 ml), (d) +CS gel without PS: implantation of CS gel beads without PS after injection of CA solution, (e) +PS suspension: injection of PS (8.3 mg) suspension with mock implantation after injection of CA solution, (f) +CS gel retaining PS: implantation of CS gel beads retaining PS (8.3 mg) after injection of CA solution.

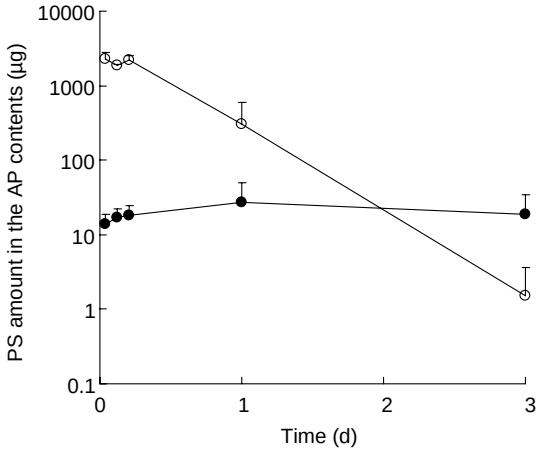


Fig. 8. Profiles of PS in the AP contents after implantation of CS gel beads retaining PS or injection of PS suspension with injection of CA solution. (●): CS gel bead retaining PS (8.3 mg), (○): PS (8.3 mg) suspension. PS retained in CS gel beads was excluded from the amount of PS in the AP contents. Data represent mean ± S.D. ($n$ = 5–10). Data were analyzed statistically using Student's $t$-test after $F$-test and Smirnov–Grubbs' outlier test ($\alpha = 0.05$).

These results indicate that controlled drug release by CS gel beads result in low PS concentrations in the blood, and this may minimize global side effects.

Fig. 10 shows the residual PS in the CS gel beads, excluding the PS in AP contents, after implantation of CS gel beads retaining PS, and the residual PS in AP

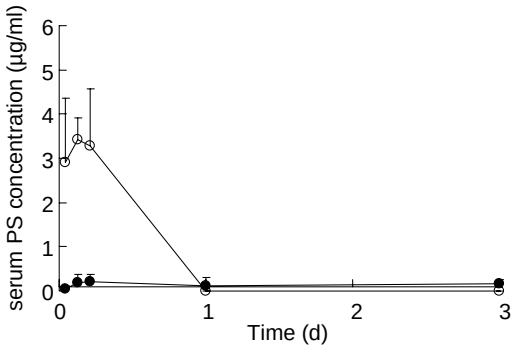


Fig. 9. Profiles of serum PS concentration after implantation of CS gel beads retaining PS or injection of PS suspension with injection of CA solution. (●): CS gel beads retaining PS (8.3 mg), (○): PS (8.3 mg) suspension. Data represent mean ± S.D. ($n$ = 4–10). Data were analyzed statistically using Student's $t$-test after $F$-test and Smirnov–Grubbs' outlier test ($\alpha = 0.05$).

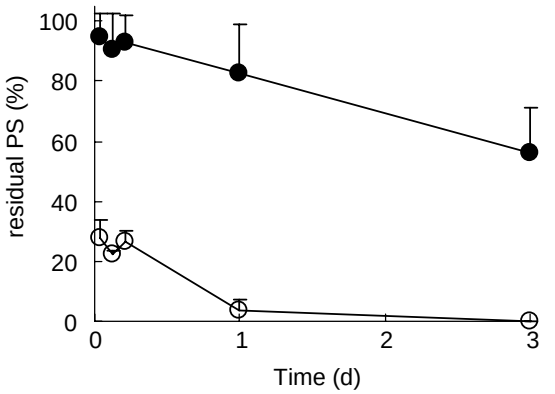


Fig. 10. Profiles of residual PS in CS gel beads after implantation of CS gel beads retaining PS and in AP after injection of PS suspension with injection of CA solution. (●): Residual PS in CS gel beads (PS in AP after release from CS gel beads was excluded) after implantation of CS gel beads retaining PS (8.3 mg), (○): Residual PS in the AP after injection of PS (8.3 mg) suspension. Data represent mean ± S.D. ($n$ = 5–22). Data were analyzed statistically using Student's $t$-test after $F$-test and Smirnov–Grubbs' outlier test ($\alpha = 0.05$).

after injection of PS suspension. Very little PS was found in AP at 3 days after injection of PS suspension, while more than 50% of the PS was retained in CS gel beads at 3 days after implantation. Furthermore, about 0.2% of the PS was present in the AP exudate, which shows that PS release from the CS gel beads was prolonged.

The present results strongly suggest that the therapeutic efficacy of PS can be improved by sustained release from CS gel beads and constant levels of PS in the target region can be maintained.

## 4. Conclusion

Sustained release of PS from CS gel beads allows the minimum effective dose to be delivered locally, and is able to prolong the duration of drug activity. This resulted in improved therapeutic efficacy against local inflammation. Furthermore, local supply of the minimum required dose may result in a reduction in side effects by minimizing transportation of the drug into the bloodstream. Control of biodegradation and drug release from CS gel beads in vivo is achieved by manipulating the properties of CS. These beads appear to be promising as an effective and biodegradable

vehicle to treat local inflammation via sustained drug delivery.

## Acknowledgements

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