Songklanakarin J. Sci. Technol.
38 (4), 421-426, Jul. - Aug. 2016

http://www.sjst.psu.ac.th

Original Article

Clinical and histopathological characteristics after subconjunctival implantation of cyclosporine-containing poly-lactic acid microfilm in normal dogs

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Received: 10 July 2015; Accepted: 1 January 2016

Abstract

The objective of the present study was to describe clinical signs and histopathology of the conjunctiva after subconjunctival implantation with poly-lactic acid microfilm (PLA-M) and cyclosporine-containing PLA microfilm (CsPLA-M) in seven healthy dogs. A randomized, double-blind implantation of a PLA-M and CsPLA-M was performed in each eye. Ocular clinical signs and Schirmer tear test (STT) levels were determined on days 1, 3, 7, 14, 28, 60 and 90 after implantation. All implanted eyes had conjunctival hyperemia after implantation which eventually resolved within 90 days. Histopathological examination of conjunctival biopsy showed mild inflammatory cell infiltration. There was no significant statistical difference of conjunctival hyperemia scores, STT values or histopathological scores between the two groups. All devices remained under subconjunctiva at 90 days after implantation. These results suggested that the device was safe and well tolerated for subconjunctival implantation in dogs.

Keywords: cyclosporine, dog, microfilm, poly-lactic acid, subconjunctival implantation

1. Introduction

Canine keratoconjunctivitis sicca (KCS) is a common ocular disease and can be defined as a progressive inflammatory condition of the cornea and conjunctiva caused by aqueous tear deficiency (Aguirre *et al.,* 1971; Barnett and Sansom, 1987; Carter and Colit, 2002; Williams, 2008). The main clinical signs are the presence of mucoid ocular discharge, conjunctival hyperemia, blepharospasm, and recurrent corneal ulceration. Other signs such as ocular pain, conjunctivitis, corneal melanosis and vascularization, including reduced vision may present depending on the stage of the

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disease. Etiology of KCS in dogs could be congenital hypoplasia, drug toxicity (sulfonamides), drug-induced (atropine), irradiation, iatrogenic (excision of nictitans gland), endocrine disorders, chronic blepharoconjunctivitis, trauma and neurologic dysfunction. The major cause of KCS in dogs is an immune-mediated disorder which occasionally associated with systemic autoimmune conditions (Carter and Colitz, 2002; Kaswan *et al.,* 1989; Matheis *et al.,* 2012; Morgan and Bachrach, 1982).

Therapeutic regimens of KCS include the use of topical anti-inflammatories, antibiotics, mucinolytics, artificial tear replacements, stimulation of natural tear production or surgical transposition of the parotid salivary duct to the conjunctival fornix. The most commonly used tear stimulator for treatment of KCS in dogs is cyclosporine A (CsA) (Izci *et al*., 2002; Morgan and Abrams, 1991; Olivero *et al.,* 1991). CsA has a selective immunosuppressive effect of specific inhibitor on the T-helper lymphocyte proliferation and infiltration of lacrimal gland acini, allowing for regeneration of the gland and return of its secretory function (Ho *et al*., 1996). Topical CsA has been reported to improve tear production for 71-86% of dogs with KCS. However, topical irritation of CsA and failure to regularly apply the medication limit the effectiveness in some canine patients.

Several types of ocular drug delivery method have been described (Ghosn *et al*., 2011; Short, 2008; Wiener and Gilger, 2010). Methods of ocular drug delivery must correlate to the site of drug target and duration of the effect needed. Sustained release ocular implants have been developed over the past decade to allow delivery of constant therapeutic level of drugs to the eye (Short, 2008; Wiener and Gilger, 2010). In general, subconjunctival or episcleral implantation is successfully used for anterior segment diseases (Gilger *et al.,* 2014; Kim *et al*., 2005). Ocular implants are reported to be useful in the treatment of chronic ocular diseases, such as equine recurrent uveitis or immune-mediated keratitis in horses (Ghosn *et al*., 2011; Gilger *et al*., 2006, 2010, 2013) and KCS or chronic superficial keratitis in dogs (Kovacova *et al*., 2013). This sustained release ocular drug delivery technology has the advantage of minimizing the effect of patient and owner noncompliance in drug administration. In addition, continuous release of the medication is typically well below toxic levels. Therefore, ocular implants benefit for therapeutic level achievement without systemic side effects. (Davis *et al.,* 2004; Wiener and Gilger, 2010)

Poly-lactic acid (PLA) is currently one of the most promising biodegradable and biocompatible polymers. It is used in diverse properties and various applications. Its biocompatibility in contact with living tissues is exploited for biomedical applications such as drug delivery system, sutures, cell scaffold and prostheses for tissue replacements such as intraocular lens, dental implant, breast implant and artificial organ for temporary or permanent assist (Cheng *et al*., 2009). PLA is a degradable biomaterial which is produced from renewable resources by fermentation of starch (Gupta *et al*., 2007). Implantation of PLA has the advantage of increasing the half-life of drugs and does not require removal (Hsu, 2007).

The objective of this study was to determine clinical signs and histopathological characteristics after subconjunctival implantation with PLA microfilm (PLA-M) and cyclosporine-containing PLA microfilm (CsPLA-M) in normal dogs.

2. Materials and Methods

All dogs received a complete physical and ocular examination together with blood examination prior to entry into the study. Only dogs that were judged to be free of any significant ocular and systemic diseases were enrolled in the study. A randomized, double-blind study of subconjunctival implantation of a PLA-M and CsPLA-M was conducted. Seven healthy mixed breed dogs at the ages ranged from 6-8 years (6.29±0.71, mean ± SD) received subconjunctival implantation of PLA-M and CsPLA-M in each eye.

2.1 Implant manufacturing

PLA microfilms for implantation were prepared from a modified emulsification-solvent evaporation technique (Gryparis *et al*, 2007). To prepare the microfilm, 2 g of PLA (Ingeo™ Biopolymer 4043D, NatureWorks LLC, USA) was dissolved in 50 ml of dichloromethane and stirred for 4 hours before being mixed with 100 mg cyclosporine (Atopica®, R.P. Schere GmbH & Co.KG., Eberbach/Baden, Germany) and dispersed in the solution using magnetic stirrer. Then the solution was casted on a 9 cm diameter sterile petri dish and dried for 72 hours. The microfilms were cut into 2 cm x 0.5 cm with a flat side for implantation. The concentration of cyclosporine in PLA was 5% (wt/wt) resulting in approximately 3.3 mg of cyclosporine loaded in each implant.

2.2 Procedures

A complete ocular examination including Schirmer tear test (STT) (Intervet Inc., NJ, USA), fluorescein stain (32 K. SUPPLY Co.,LTD, Bangkok, Thailand), slit lamp biomicroscopy (model SL-15, Kowa Optimed Co. Ltd. United Kingdom), rebound tonometer (TonoVet®, Icare Finland Oy, Helsinki, Finland), and indirect ophthalmoscopy (Welch Allyn, Skaneatales, NY, USA) was performed in all dogs before device implantation.

The surgical implantation was performed in all dogs under general anesthesia. All dogs were premedicated and induced with intravenous administration of 0.3 mg/kg diazepam (GPO, Thailand) and 4 mg/kg propofol (Anepol®, Hana Pharm, Korea), respectively. The anesthesia was maintained throughout the operation with 2.5-3 % isoflurane (Aerrane®, Baxtex Healthcare Co., Ltd Bangkok, Thailand). After surgical aseptic preparation, a 5 mm incision was made through the dorsal aspect of the bulbar conjunctiva. A pocket was formed in subconjunctival space parallel to limbus. One

implant was randomly selected and inserted into this space. Then, the conjunctiva was closed with a single interrupted 6-0 Polydioxanone (PDS) absorbable suture. The other eye was implanted with a different PLA film type either with or without cyclosporine by the same procedure. An Elizabethan collar was used to prevent eye scratching. A combination of neomycin sulfate and dexamethasone (Dex-oph®, Sang Thai Medical, Bangkok, Thailand) was applied postoperatively for 7 to 14 days depending on clinical signs. An anti-inflammatory agent, carprofen (Rimadyl®, Zoetis Limited, Bangkok, Thailand) was orally administered at the dosage of 4.4 mg/kg once a day for 5 consecutive days after implantation.

2.3 Clinical examination

Degrees of conjunctival hyperemia were scored on pictures as follow; 0 if absent, 1 if mild, 2 if moderate and 3 if severe (modified from Barachetti, *et al*, 2015). Other signs such as blepharospasm, ocular discharge, corneal neovascularization and corneal opacity were investigated. Schirmer tear tests, dazzle reflex, menace response, pupillary light reflex and fluorescein stain were also examined. Fundus was investigated with an indirect ophthalmoscopy. Follow-up examinations were performed post-operatively at 1, 3, 7, 14, 28, 60 and 90 days. Pictures of clinical signs were recorded by a DSLR camera (Nikon D90, Nikon Inc., Thailand) with a macro lens (Tokina 100F 2.8D macro, Tokina Co., Ltd., Japan).

2.4 Histopathological examination

Bilateral conjunctival biopsy of the bulbar conjunctiva at the area of implantation of seven healthy dogs (14 eyes) was performed on day 28 and 90 post-operatively at the size of 4x4 mm. The conjunctival tissues were immersed in 10% buffered formalin for histopathology. Degrees of inflammation were scored from 0 to 4 (Table 1).

2.5 Data analysis and statistical methods

Data of STT, severity of conjunctival hyperemia and histopathological scores were analyzed and compared between PLA-M and CsPLA-M using repeated measure ANOVA method. A value of $P<0.05$ was considered as statistical significance. All data were analyzed by commercial software (NCSS, 2007).

3. Results

All dogs tolerated the devices well. None of dogs showed signs of blepharospasm or corneal opacity after implantation. Three dogs had no ocular discharge at all follow up periods whereas two had bilateral mild mucous ocular discharge at the median canthus for one or two weeks and the other two had bilateral mild ocular discharge until day 60.

All dogs had bilateral conjunctival hyperemia after implantation. Three dogs had unilateral severe conjunctivitis within the first week after implantation. Two of these eyes were implanted with CsPLA-M and the other one was implanted with PLA-M. The other surgical eyes had mild or moderate conjunctivitis. Severe conjunctivitis in three eyes was markedly reduced within the first week. At 28 days postoperation, four eyes implanted with CsPLA-M and three eyes implanted with PLA-M still had conjunctival hyperemia, whereas conjunctival hyperemia in the other seven eyes was completely resolved (Figure 1). Only one eye which was implanted with PLA-M still had mild conjunctival hyperemia at day 90 post-operation. Scores of conjunctival hyperemia were slightly higher in eyes implanted with CsPLA-M. However, there were no significant differences of scores of conjunctival hyperemia between the two groups (Figure 2). The devices were retained in every eye on day 90. Schirmer tear test values were not statistically different in any measurement between the two groups (Figure 3).

Other ocular abnormality was not found during follow up period in any eye. All response, reflexes, intraocular pressures and fundic appearance were also normal and fluorescein staining were negative in all eyes at every follow up period.

Histopathological examination of conjunctival biopsy on day 28 and 90 after implantation showed mild to moderate chronic inflammatory cell infiltration and congestion in both eyes (Figure 4). The inflammation scores of the conjunctiva

Table 1. Lesion score of the inflammation of conjunctival tissue.

lesion score	criteria
0	no microscopic lesions
1	congestion
2	edema and congestion of the conjunctiva
3	infiltration of inflammatory cells, e.g. neutrophils and edema of the conjunctiva
4	infiltration of inflammatory cells, e.g. neutrophils, edema of the conjunctiva and denudation of the conjunctival epithelium

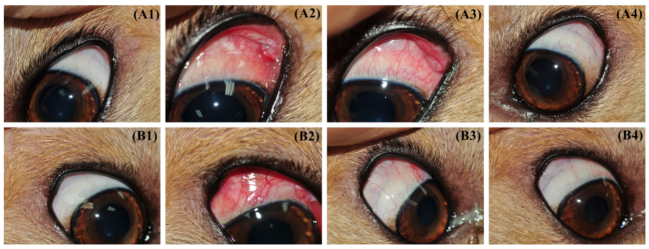

Figure 1. Pictures of the eyes before and after PLA (A1-A4) and cyclosporine-containing PLA (B1- B4) microfilm implantation on day 0 (A1, B1), day 3 (A2-B2), day 7 (A3-B3) and day 28 (A4-B4); Note the inflammation was observed on day 3 and day 7 post-operation and eventually resolved within 28 days.

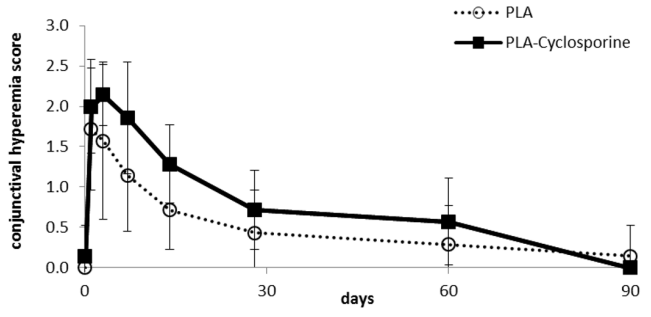

Figure 2. Severity of conjunctival hyperemia before and after the implantation of PLA (dotted line) and cyclosporine-containing PLA microfilm (black line).

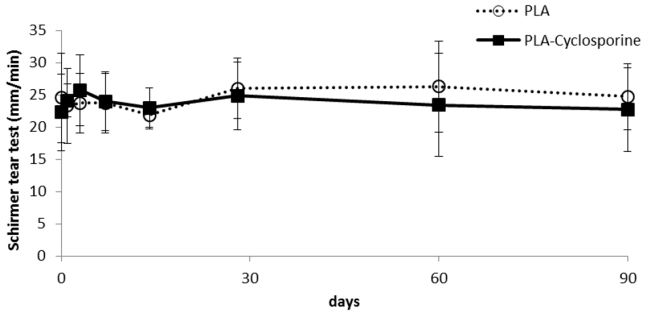

Figure 3. Schirmer tear test (STT) values (mean ± SD) before and after the implantation of PLA (dotted line) and cyclosporine-containing PLA microfilm (black line).

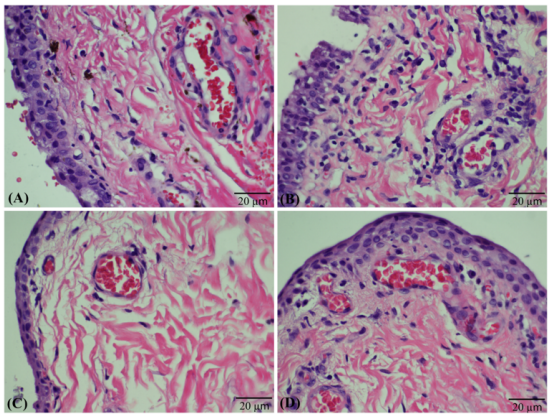

Figure 4. Severity of histopathologic lesions: After implantation for 28 days with PLA (A) and cyclosporine-containing PLA (B) microfilms and for 90 days with PLA (C) and cyclosporine-containing PLA (D) microfilms. Score 2 for A and B and Score 1 for C and D. All conjunctival tissues were stained with hematoxylin and eosin (H & E) 600X. Note: Scale bar 20 µm

in dogs with PLA-M and CsPLA-M on day 28 after surgery were 2.00±0.82 (mean ± SD) and 2.00±0.58 respectively. While the score on day 90 were 1.71±0.75 and 1.86±0.90 respectively. There were no significant differences of histopathological scores between the two groups at the two study periods. No evidence of infection was found in any of the tissue sections.

4. Discussion

In this preclinical evaluation, the subconjunctival cyclosporine-containing PLA microfilm implant appeared to be safe for dogs. It caused only mild to moderate clinical inflammation in the majority of the implanted eyes, which is a typical complication after general conjunctival surgery. Severe conjunctival hyperemia in three eyes was possibly caused by surgical technique because it occurred in both PLA-M and CsPLA-M implantation and markedly improved within one week post-operatively. Conjunctival hyperemia scores were slightly higher in the eyes implanted with CsPLA-M. The reason might be the reaction to cyclosporine itself or the slightly higher thickness of the materials containing cyclosporine. Serious complications such as tissue erosion, implant migration and implant extrusion (Nguyen, 2004) were not found in this study. This was the first time that cyclosporine-containing PLA microfilm was used for subconjunctival drug delivery system.

Episcleral implantation by episcleral silicone matrix cyclosporine has successfully been used for treatment of KCS in dogs. (Barachetti *et al*., 2015). However, silicone is a non-biodegradable material while PLA is a degradable material which had the advantage of steady, controlled release of drug during long periods of time (Kim *et al*., 2005; Davis *et al*., 2004; Short, 2008; Wiener and Gilger, 2010). PLA has been used in rabbits for drug delivery system in forms of PLA derivatives such as poly (lactide-co-glycolide) copolymer intravitreal implant containing dexamethasone with no toxicity to normal rabbit retina and no effect on intraocular pressure. Intravitreal drug concentration remained within therapeutic range up to 8 week period of evaluation (Fialho *et al*., 2006). Poly (lactide)/monomethoxy-poly (ethyleneglycol) nanoparticles, another PLA derivative, has been developed as a drug delivery system at the subconjunctival space for latanoprost acid in rabbits with no adverse effect to the eye and intraocular pressure (Giarmoukakis *et al*., 2013). In general, PLA derivatives were invented to increase the degradation rate. However, KCS is a chronic disease for which lifelong treatment is usually required. Therefore PLA, instead of PLA-derivatives was chosen in this study.

No statistical significant difference of histopathological scores and clinical signs of PLA implantation between the two groups in this study confirmed the safety of CsPLA-M implantation in dogs. Further improvement is needed for the adjustment of the implant size and the amount of cyclosporine to increase both drug load and the surface releasing area. These will help enhance the drug release rate from this matrix device (Davis *et al*., 2004).

In conclusion, to our knowledge this is the first study of a delivery system of the subconjunctival biodegradable implantation with CsPLA-M. CsPLA-M implantation was safe, well-tolerated with no sign of serious adverse tissue

reaction observed. Studies focusing on the efficacy and optimization of this delivery system with regard to its efficiency and long-term safety are considered necessary. Further studies are needed to assure its clinical application for KCS management.

Acknowledgements

We gratefully acknowledge the help of Dr. Metita Sussadee and all staffs at Ophthalmology clinic, Kasetsart University Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, for their assistance in this study. The authors also acknowledge Kongkiat Kongsuwan and Suthawan Buchatip for technical assistance in the PLA manufacturing.

This study was supported by Intervet Research Foundation.

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