

Dermal microdialysis in the dog: *in vivo* assessment of the effect of cyclosporin A on cutaneous histamine and prostaglandin D₂ release

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Abstract

Dermal microdialysis, a relatively noninvasive technique, allows investigation of the changes in cellular mediators released during cutaneous allergic responses. This technique was used to evaluate the effect of cyclosporin A, an immunosuppressive drug used for treatment of canine atopic dermatitis, on the cutaneous release of two pro-inflammatory mediators following intradermal allergen challenge. Four beagle dogs spontaneously sensitized to *Ascaris suum* were treated for 1 month with oral cyclosporin A. At days 0, 15 and 30 of the treatment, dialysis probes were inserted into the skin of the back, and 20 µL of *A. suum* antigen was injected intradermally at each site. At timed intervals, dialysate was collected and assayed for histamine and prostaglandin D₂ and the wheal area was measured. Mean histamine concentration and wheal area were significantly lower at days 15 and 30 of treatment, compared with day 0. However, prostaglandin D₂ concentration was not significantly reduced. The inhibition in histamine release after intradermal challenge, by cyclosporin, confirms its anti-inflammatory action in the dog. Dermal microdialysis provides a useful tool for investigating canine allergic reactions and their modulation by drugs.

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Introduction

Microdialysis in human dermatological research has permitted *in vivo* study of the cutaneous interstitial space.¹ This technique enables water-soluble molecules in the extracellular fluid compartment to diffuse through a semipermeable dialysis membrane inserted into the dermis, allowing changes in mediator concentration during an inflammatory or allergic cutaneous reaction to be followed.^{2,3} Recently, microdialysis has been used in dogs⁴ to study the mediator basis of the early phase of the wheal and flare responses induced by intradermal injection of allergen. Cyclosporin A (CsA), an immunosuppressive drug commonly used in human medicine, has been recently reported in the treatment of a number of inflammatory and allergic skin conditions in dogs, including canine atopic dermatitis.^{5,6} Oral CsA reduces skin lesions and pruritus in dogs with atopic dermatitis.⁷

The cellular effects of CsA are mediated by an intracellular calcium-dependent pathway. More specifically, CsA binds to a cytosolic cyclophilin and inhibits a protein, calcineurin, necessary for gene transcription and cell activation.⁸ By this means, CsA is able in humans to inhibit the activation of numerous cell types, including T cells, eosinophils and mast cells.^{9,10}

Some *in vitro* studies performed in canine cells have shown an inhibitory effect of CsA on the release of mast cell mediators like histamine¹¹ and also on the induction of apoptosis in a canine T-lymphocyte cell line.¹² However, the effect of CsA on the release of pro-inflammatory mediators has not been studied *in vivo* during an allergic reaction in the dog.

The objectives were thus to evaluate the efficacy of dermal microdialysis in the study of inflammatory mediators in dog skin and thereby to assess the effect of CsA on histamine and prostaglandin D₂ (PGD₂) release, after an intradermal antigen challenge in *Ascaris suum*-hypersensitive dogs. The anti-inflammatory response of CsA in the wheal area induced by the intradermal antigen challenge was also evaluated.

Materials and methods

Animals

Four female beagle dogs (ages 1–3 years old), spontaneously sensitized to *A. suum*, were used. No drugs or additional treatments were given during the study, except sedatives and anaesthetics, administered prior to the microdialysis experiments described later. The study was approved by the Animal Research Ethical Committee of the University. All animals were adopted at the end of the study.

Part of the results was presented at the 19th Annual Congress of the ESVD-ECVD in Tenerife, Spain in September 2003.

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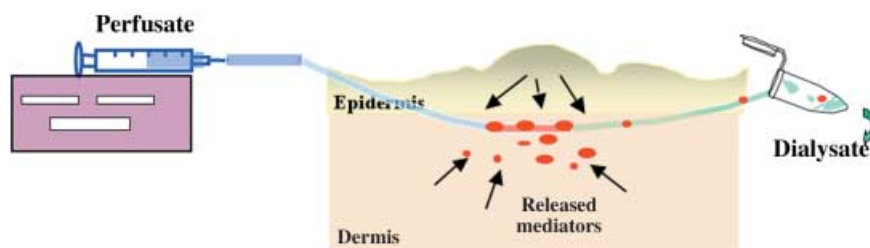


Figure 1. Schematic drawing of the principle of the dermal microdialysis. Sterile phosphate-buffered solution is infused at a rate of $5 \mu\text{L min}^{-1}$ through a dialysis probe 20 mm long lying parallel with the epidermis and the outflow from the tubes (which includes dialysate of released mediators) is collected into plastic vials for analysis.

Dermal microdialysis

The dogs were sedated by intramuscular injection of buprenorphine (Buprex^R, Schering-Plough, Madrid, Spain) (0.01 mg kg^{-1}) and midazolam (Dormicum^R, Roche, Madrid, Spain) (0.2 mg kg^{-1}), and then anaesthetized with intravenous propofol (Propofol Lipuro, Braun, Barcelona, Spain) at an induction dose of 4 mg kg^{-1} and a maintenance dose of 1 mg kg^{-1} .

Linear flow microdialysis probes were constructed from cylindrical hollow porous membranes with a 5 kDa molecular mass cut off and external diameter of 0.22 mm (Focus 90H Hemophan Hollow Fibre Dialyser, NMC, Rockleigh, NJ, USA). The membranes were strengthened with a steel wire and glued into a short length of Portex tubing (Scientific Laboratory Supplies Ltd, Nottingham, UK), as described previously.¹³ Two pairs of probes were inserted into the upper dermis of the dog trunk using a 23-gauge-guide cannula. Each probe had an effective dialysis length of 20 mm and was inserted to lie parallel to the epidermis (Fig. 1). The probes in each pair were 3 mm apart and the distance between each pair of probes was 5 cm (Fig. 2).

After 1 h for recovery from insertion trauma, the probes were connected via a length of Tygon tubing (Cole-Parmer, Huco Erlross, Madrid, Spain) to a syringe mounted in a microinfusion pump (KDS220, KDSscientific, Holliston, MA, USA) and perfused with sterile phosphate-buffered solution (PBS) (Gibco, Invitrogen, Barcelona, Spain) at a rate of $5 \mu\text{L min}^{-1}$. The outflow from the tubes (dialysate) was collected into plastic vials (Figs 1 and 2). Baseline dialysate samples were collected every 5 min for 20 min prior to antigen challenge.

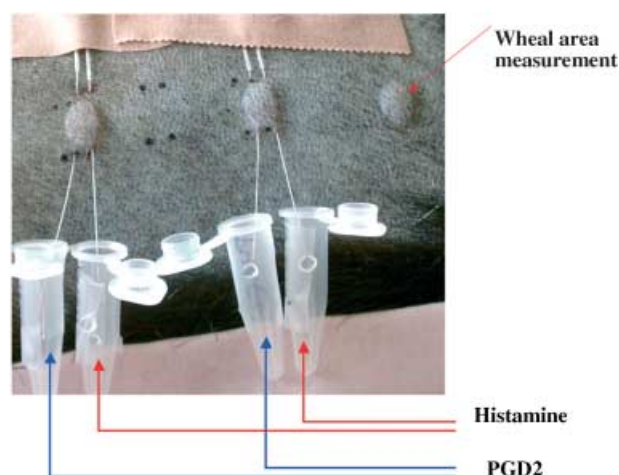


Figure 2. Photograph of one representative dermal microdialysis experiment. Two pairs of microdialysis probes (duplicates) were inserted in the skin. One fibre of each pair was used to collect histamine and the other for PGD_2 recovery after antigen intradermal injection of $20 \mu\text{L}$ of *Asc S 1* antigen (0.1 mg mL^{-1}). The third antigen challenge site (on the right) was used for wheal area measurement.

The intradermal injection of the *Asc S 1* antigen was performed as follows. Twenty microlitres of *Asc S 1* (0.1 mg mL^{-1}) was injected between the two microdialysis probes using a 27-gauge needle and a Hamilton syringe. After intradermal injection of allergen, dialysate was collected to assess released mediators (histamine and PGD_2). All samples were immediately frozen and stored at -80°C .

Experimental design

A preliminary experiment was performed to confirm the sensitivity of the dogs to *A. suum* and to establish the appropriate concentration of allergen required for further studies. This experiment was repeated every 2 months, for a period of 6 months, to confirm that responses to *A. suum* did not vary with time. Three pairs of dialysis probes were inserted, as previously described, in three adjacent sites of the skin. Each dog was challenged with intradermal injections of $20 \mu\text{L}$ of *A. suum* antigen (*Asc S 1*) (Greer Laboratories, Lenoir, NC, USA) at three concentrations (0.001 , 0.01 and 0.1 mg mL^{-1}), and samples were obtained over a 40-min period (Fig. 3). A rise in the histamine levels in the dialysate showed that all the dogs were sensitive to *A. suum*. A concentration of 0.1 mg mL^{-1} of *Asc S 1*, at which maximum levels of histamine were recovered, was chosen for the studies with CsA.

Two months after the last allergen injection of the preliminary experiment, the same animals were treated with oral CsA (Sandimmune-Neoral, Novartis Animal Health, Basel, Switzerland) at a dose of $5 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 30 days. At days 0, 15 and 30 of treatment, dialysis probes were inserted into two adjacent sites (duplicate) on the dorsal skin of each dog (two probes were inserted per site). Two hours after CsA administration, $20 \mu\text{L}$ of *A. suum* antigen (0.1 mg mL^{-1}) was simultaneously injected intradermally at each site. Dialysate from one of the probes in each pair was collected every 5 min for up to 20 min to assess histamine, and dialysate from the other probe was collected for up to 50 min at 10 min intervals to assess PGD_2 . Interval times were chosen to accommodate the requirements of the assays.

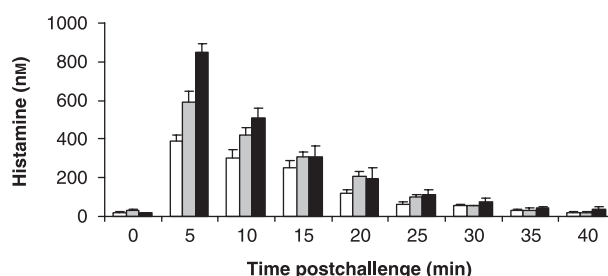


Figure 3. Histamine concentrations collected in microdialysis fluid every 5 min over a 40-min period after intradermal injection of $20 \mu\text{L}$ of *Asc S 1* antigen at a concentration of 0.001 mg mL^{-1} (white bars), 0.01 mg mL^{-1} (grey bars) and 0.1 mg mL^{-1} (black bars). There was a significant rise in histamine level in the dialysate ($P < 0.01$) within 5 min that gradually declined to basal level at 35 min. Mean $\pm$ standard error.

The wheal area was also measured at a separate *Asc S 1*-injected site (Fig. 2). Venous blood samples (2 mL) were taken to assess the total serum IgE levels in each dog.

Mediator determination

Histamine concentrations were assessed using a competitive immunoassay (IMMUNOTECH, Marseille, France) with a sensitivity of 1 nM. For PGD₂ measurement, a commercial immunoassay with a detection limit of 1.95 pg mL⁻¹ was used (Cayman Chemical, Ann Arbor, Michigan, USA). All samples were assayed in duplicate.

Wheal area

Wheal area was measured by planimetry 15 min after allergen injection at a third separate allergen-injected site 4 cm from the other two sites. An acetate sheet was placed on the skin over the reaction and the outline of the wheal traced. Reaction area was calculated from the tracing using an image analyser program, MIP 4 ADVANCED (Microm España CID, Madrid, Spain). To minimize test variability all measurements were performed in triplicate by the same technician.

Total IgE determination

Total serum IgE was determined at days 0, 15 and 30 using a canine IgE ELISA assay (Bethyl Laboratories, TX, USA).

Statistics

Results are expressed as the arithmetical mean $\pm$ standard error (mean $\pm$ SEM) of four experiments. The effects of treatment were tested for significance using two-way analysis of variance (ANOVA), in which wheal area and histamine release were the response variables and dog and time the row and column factors. A probability of $P < 0.05$ was taken to be statistically significant.

Results

CsA study – histamine release

Asc S 1-induced histamine release 0, 15 and 30 days after treatment with CsA is shown in Fig. 4. The mean basal histamine concentration in the dialysate prior to antigen injection (day 0) was 10.4 ± 2.9 nM. Following injection,

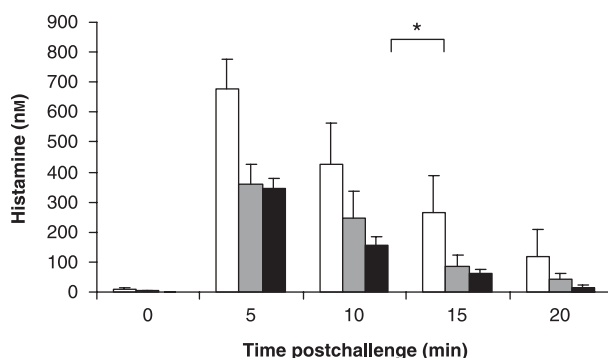


Figure 4. Histamine concentrations collected in microdialysis fluid after intradermal injection of 20 μ L of *Asc S 1* antigen (0.1 mg mL⁻¹) at day 0 (white bars), 15 (grey bars) and 30 (black bars) of oral treatment with CsA (5 mg kg⁻¹ day⁻¹). Mean $\pm$ standard error. Histamine level in the dialysate rose significantly ($P < 0.01$) within 5 min in every instance and gradually declined towards basal level. However, after 15 and 30 days, the peak concentrations of histamine found after 5 min of sampling, were significantly lower than those found at 0 days (* $P < 0.01$).

histamine levels increased significantly ($P < 0.01$) to a maximum of 677 ± 98 nM in the first 5 min collection, and gradually declined to basal level at 20 min. However, after 15 and 30 days, the peak concentrations of histamine found after 5 min of sampling time, were significantly lower than those found at 0 day (* $P < 0.01$).

CsA study – PGD₂ release

Asc S 1-induced PGD₂ release 0, 15 and 30 days after treatment with CsA is shown in Fig. 5. PGD₂ concentration in the dialysate increased within 10 min of intradermal antigen injection and gradually decreased over the following 50 min. Baseline had still not been reached by this time. A peak concentration of PGD₂ at day 0 (277 ± 71 pg mL⁻¹) was found 10 min after *Asc S 1* injection, but no significant differences in PGD₂ levels were found with respect to 15 and 30 days of treatment at any of the other sampling times.

Wheal area

The wheal areas induced in the skin after intradermal injection of *Asc S 1* antigen before and after 15 and 30 days of CsA treatment are shown in Table 1. After 15 and 30 days of treatment with CsA, wheal areas, measured at 15 min after challenge, were significantly attenuated ($P < 0.028$, ANOVA contained data from all dogs at all times). There was a significant correlation between peak histamine levels and wheal areas ($P < 0.008$, $r^2 = 0.52$), as shown in Fig. 6.

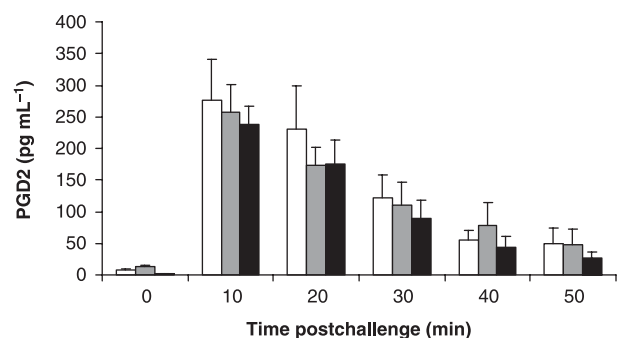


Figure 5. Prostaglandin D₂ concentrations collected in microdialysis fluid after intradermal injection of 20 μ L of *Asc S 1* antigen (0.1 mg mL⁻¹) at day 0 (white bars), 15 (grey bars) and 30 (black bars) of oral treatment with CsA (5 mg kg⁻¹ day⁻¹). Mean $\pm$ standard error. Note the increased PGD₂ concentration in the dialysate within 10 min of injection ($P < 0.01$) and gradual decrease over the following 50 min. No significant differences in PGD₂ levels were found with respect to days of treatment at any of the sampling times.

Table 1. Wheal areas obtained 15 min after intradermal injection of 20 μ L of *Asc S 1* antigen (0.1 mg mL⁻¹) at days 0, 15 and 30 of oral treatment with CsA (5 mg kg⁻¹ day⁻¹). Mean of three repeated readings $\pm$ standard error. Treatment with CsA significantly attenuated the wheal areas ($P < 0.028$, ANOVA contained data from all dogs at all times)

Dog code	Wheal areas (mm ²)		
	day 0	day 15	day 30
J44	132.1 $\pm$ 11.9	115.0 $\pm$ 4.1	76.8 $\pm$ 2.0
J34	173.2 $\pm$ 7.8	115.4 $\pm$ 8.3	86.5 $\pm$ 3.9
GZG4	91.7 $\pm$ 10.9	92.7 $\pm$ 3.9	85.7 $\pm$ 6.3
1J02	125.8 $\pm$ 8.7	79.2 $\pm$ 4.4	78.2 $\pm$ 4.1

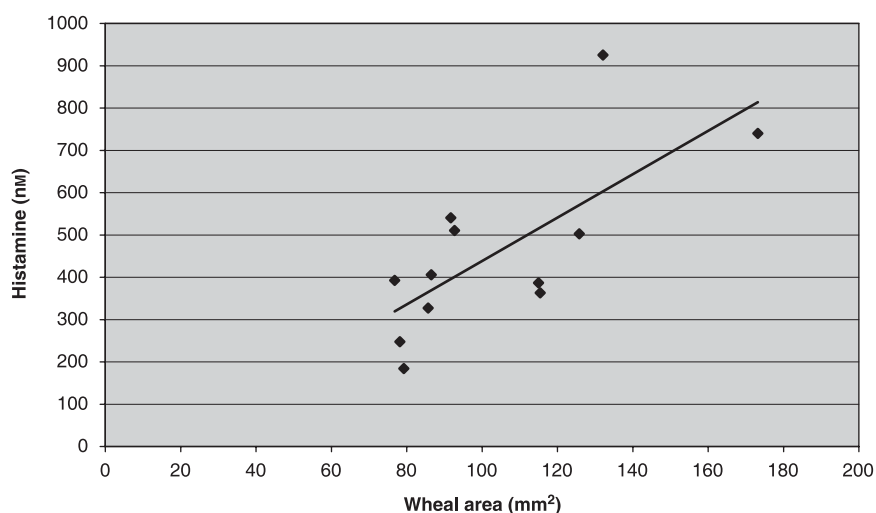


Figure 6. Correlation between maximum histamine concentrations (nm) observed 5 min after antigen injection and the corresponding wheal areas (mm²) ($r^2 = 0.52$).

Total IgE

All dogs showed a high mean serum IgE level ($2.93 \pm 0.51 \mu\text{g mL}^{-1}$) before treatment and this was not significantly reduced over the 30-day treatment period ($2.61 \pm 0.36 \mu\text{g mL}^{-1}$).

Discussion

The *in vitro* inhibitory effect of CsA on histamine release has been reported in human¹⁴ and canine¹¹ dermal mast cells *in vitro*. This study demonstrated that mean histamine concentration released in the skin of dogs was also significantly reduced *in vivo* by CsA treatment. This could be explained by a decrease in mast cell numbers as has been reported in the mouse.¹⁵ If this was the mechanism, a parallel reduction of PGD₂ release would have been expected. However, PGD₂ recovery remained constant throughout the duration of treatment. An alternative explanation is a decrease in histamine release from mast cells, CsA having been shown to inhibit receptor-mediated exocytosis.¹⁶ Interestingly, these studies, which were performed in a rat basophilic leukaemia cell line, also showed that while degranulation was attenuated, CsA did not inhibit the biochemical events leading to eicosanoid synthesis and secretion. This would explain the lack of effect of CsA treatment on the recovery of PGD₂ in this study.

In parallel to the cutaneous histamine inhibition, a significant decrease on the skin wheal area was observed during CsA treatment. There are few studies in dogs reporting the effect of oral CsA on intradermal tests, although a study in atopic dogs showed a weak reduction on wheal size after 45 days of CsA oral treatment.¹⁷ Studies in humans have shown that histamine is the major mast cell mediator responsible for the development of the wheal, there being a strong correlation between the concentration of histamine recovered by microdialysis and the wheal area.¹⁸ The finding of a significant correlation between histamine levels and wheal area in this study suggests that wheal development in dogs is also principally histamine dependent.

The finding that treatment with CsA did not alter IgE levels is consistent with a previous study in dogs.¹⁹

In conclusion, the results show that CsA can inhibit the release of histamine and subsequent wheal formation

in the skin of sensitized dogs, after intradermal antigen challenge. By this dynamic sampling technique it is possible, therefore, to carry out different types of investigations such as studies on the pathogenesis and pharmacological control of allergic cutaneous reactions. This application appears to be of value for evaluation of mediator release (early or late phase reactions) and their role in control of these reactions.

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Résumé La microdialyse dermique, une technique relativement peu invasive, permet l'étude des modifications des médiateurs cellulaires libérés pendant les réactions cutanées allergiques. Cette technique a été utilisée pour évaluer les effets de la ciclosporine A, un médicament immunomodulateur utilisé pour le traitement de la dermatite atopique canine, sur la libération cutanée de deux médiateurs pro-inflammatoires à la suite d'un test intradermique.

Quatre Beagle sensibilisés à *Ascaris suum* ont été traités pendant un mois avec la ciclosporine A par voie orale. A J0, J15 et J30, des sondes de dialyse ont été mises en place dans la peau du dos et 20 µL d'antigène d'*Ascaris suum* ont été injectés par voie intradermique à chaque site. Les dialysats ont été prélevés et l'histamine et la prostaglandine D2 ont été mesurées, ainsi que la taille de la plaque ortiée.

Les concentrations moyennes en histamine et la taille de la plaque ortiée étaient significativement plus faibles à J15 et J30 en comparaison avec J0. Cependant les taux de prostaglandine D2 n'étaient pas significativement diminués. L'inhibition de la libération d'histamine confirme l'effet anti-inflammatoire de la ciclosporine chez le chien.

La microdialyse dermique représente une technique intéressante pour évaluer les réactions allergiques cutanées chez le chien et leur modulation par les médicaments.

Resumen La microdiálisis dérmica, una técnica relativamente poco agresiva, permite la investigación de los cambios en mediadores celulares secretados durante las respuestas alérgicas cutáneas. Se utilizó esta técnica para evaluar el efecto de la ciclosporina A, un fármaco inmunosupresor utilizado para el tratamiento de la dermatitis atópica, en la producción de dos mediadores de inflamación tras la re-exposición intradérmica a un alérgeno.

Cuatro perros Beagle sensibilizados de manera espontánea a *Ascaris suum* fueron tratados durante un mes con Ciclosporina A. En los días 0, 15 y 30 del tratamiento las agujas de diálisis fueron insertadas por vía intradérmica en predeterminadas localizaciones. Se recogió un dializado a diferentes intervalos y se evaluó la presencia de histamina y prostaglandina D₂ así como el área de la pápula.

La concentración media de histamina y el área de pápula fueron significativamente menores en los días 15 y 30 de tratamiento, en comparación con el día 0. Sin embargo la concentración de prostaglandina D₂ no se redujo de forma significativa. La inhibición de la secreción de histamina tras la re-exposición intradérmica por la ciclosporina A confirma su actividad antiinflamatoria en el perro. La técnica de microdiálisis dérmica aporta una herramienta de utilidad para investigar las reacciones alérgicas caninas y su modulación farmacológica.

Zusammenfassung Die dermale Mikrodialyse, eine verhältnismässig nicht-invasive Technik, ermöglicht die Untersuchung von Veränderungen zellulärer Mediatoren, die bei kutanen allergischen Reaktionen freigesetzt werden. Diese Technik wurde verwendet, um die Wirkung von Cyclosporin A, einem immunsuppressiven Medikament, welches zur Behandlung von caniner atopischer Dermatitis eingesetzt wurde, auf die kutane Freisetzung von zwei proinflammatorischen Mediatoren nach intradermaler Allergen-Provokation zu evaluieren. Vier Beagles, die spontan auf *Ascaris suum* sensibilisiert waren, wurden einen Monat lang mit Cyclosporin A behandelt. An den Tagen 0, 15 und 30 der Behandlung wurden Dialysesonden in die Rückenhaut eingeführt und 20 µL von *Ascaris suum* Antigen intradermal an jeder Stelle injiziert. Nach bestimmten Zeitintervallen wurden Dialysate gesammelt und auf Histamin und Prostaglandin D₂ Gehalt

untersucht, sowie die Schwellung gemessen. Die durchschnittliche Histaminkonzentration sowie die Schwellung waren an den Behandlungstagen 15 und 30 im Vergleich zu Tag 0 signifikant niedriger. Die Prostaglandin D₂ Werte waren jedoch nicht signifikant vermindert. Die Hemmung der Histaminfreisetzung durch Cyclosporin nach intradermaler Provokation bestätigt seine anti-inflammatorische Aktivität beim Hund. Die dermale Mikrodialyse stellt ein nützliches Hilfsmittel dar, um die allergischen Reaktionen des Hundes und ihre Modulation durch Medikamente zu untersuchen.