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Lack of effects on lymphocyte function from chronic topical ocular cyclosporine medication: a prospective study

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Abstract

Aim Topical cyclosporine has been widely used in the treatment of canine keratoconjunctivitis sicca without apparent documented clinical side effects. Thus the finding of reduced lymphocyte proliferation in animals treated with the drug at a concentration of 2% was both surprising and concerning. This study aimed to repeat the previous study and to compare the systemic effects of 2% cyclosporine in corn oil and 0.2% topical cyclosporine ointment (Optimmune, Intervet-Schering Plough, Welwyn, UK).

Methods Twenty dogs treated with Optimmune or with topical 2% cyclosporine in corn oil where previous treatment with Optimmune had failed were included in this study. Blood samples were taken at the time of first evaluation and at 1, 3 and 6 months of treatment to provide a biochemical and hematological health evaluation of the dogs and at each examination to measure circulating levels of cyclosporine and to obtain a lymphocyte population with which to determine a mitogen stimulation index (MSI) on treatment with phytohaemagglutinin-P (PHA) and conconavlin A (con-A). Levels of circulating cyclosporine were measured with an enzyme-multiplied immunoassay method and also the more sensitive quantification technique of mass spectroscopy (MS).

Results No blood samples contained over 15 ng/ml cyclosporine, the lower limit of detection using the radioimmunoassay or the enzyme-multiplied immunoassay technique. Positive control samples taken from dogs treated with oral cyclosporine for anal furunculosis showed measurable levels in blood, demonstrating that the technique worked. Mean MSI values at 0, 1, 3 and 6 months of treatment were 10.2, 11.4, 11.6, and 10.5 for dogs treated with 0.2% cyclosporine and 10.4, 11.9, 11.7, and 12.9 for dogs treated with 2% cyclosporine. Mitogen stimulation index values were not statistically different between the first examination and any subsequent examination time-point.

Conclusions The findings of the study contradict those of the previous studies. No change in lymphocyte stimulation index was noted, neither were significant blood levels of cyclosporine documented after topical administration of either 0.2% or 2% cyclosporine. This study shows that topical cyclosporine is safe to use in the canine eye in line with the drug's safety record in this therapeutic regime over the past 20 years since its first use.

Key Words: absorption, cyclosporine, eye, lymphocyte, side effects, systemic

INTRODUCTION

Since the discovery of the lacrimogenic effects of topical cyclosporine in the late 1980s,[1] the drug has been used in a large number of dogs, for treatment of both keratoconjunctivitis sicca (KCS),[2] and chronic superficial keratitis (CSK).[3] This second indication for topical cyclosporine treatment is related not to lacrimogenic activity, but to the classic specific T-cell immunosuppression, the initial reason for cyclosporine use in transplant patients.[4] Systemic clinical immunosuppressive side effects have not been reported in any of the thousands of dogs treated in the UK or the USA with topical therapy. This was difficult to correlate with the findings of one study in which long term topical treatment with 2%

cyclosporine resulted in reduction in lymphocyte activity, as measured by mitogen stimulation index (MSI).[5,6] This study aimed to measure circulating levels of cyclosporine and determine lymphocyte mitogen stimulation indices in dogs treated with topical 2% cyclosporine in corn oil and in dogs in which the licensed 0.2% cyclosporine ointment formulation (Optimmune, Intervet Schering-Plough, Welwyn, UK) was used.

MATERIALS AND METHODS

Twenty dogs included in the study were referred after diagnosis of being affected by KCS, CSK or lymphocytic plasmacytic conjunctivitis. Dogs were assigned to one of two treatment groups. Animals in the first group were treated with 2% cyclosporine in corn oil (Corn Oil, Mazola) while those in the second group were treated with 0.2% cyclosporine in an ointment formulation (Optimmune, Intervet Schering-Plough). Animals assigned to the 2% treatment group were those in which a previous treatment with 0.2% cyclosporine ointment had been ineffective, since the Cascade system in the UK does not permit the use of a non-licensed drug unless the licensed product has been shown to be ineffective.[7] This did mean that a fully random assignment was not possible, as discussed further below, but this is not considered to have affected the results of the study.

From an ethical perspective this study was conducted under the auspices of the Veterinary Surgeons Act (1966) rather than the Animal Scientific Procedures Act (ASPA 1996) given that the findings were of direct relevance to each animal in the study. Had the findings of the previous reports been borne out in this study and deleterious effects on systemic immune function been documented, alteration in the treatment regime would have been necessary in individual animals. Thus blood sampling of these animals was undertaken as a veterinary procedure rather than an ASPA scientific procedure.

Dogs were examined ophthalmoscopically using direct and indirect ophthalmoscopy and slit lamp biomicroscopy at 0, 1, 3 and 6 months of treatment at which time blood samples were taken into both EDTA and heparin anticoagulant. A routine hematology and biochemistry panel was performed in each dog to ensure clinical health. EDTA-anticoagulated plasma samples were frozen at −20 °C and stored at this temperature prior to cyclosporine assay. Lymphocytes were separated from heparin-anticoagulated samples by standard Ficol-Hypaque density gradient centrifugation as previously reported.[8] After separation, cells were washed and resuspended twice in RPMI 1640 (Sigma Chem Co, Poole, UK) with 10% bovine fetal serum followed by a single wash and resuspension in Hanks medium (Sigma Chem Co). Lymphocyte activity was measured by the use of a standard mitogen proliferation assay using phytohaemagglutinin-P (PHA) and conconavlin A (con-A) as previously reported.[9] Two hundred microliters of a lymphocyte suspension at a concentration of 1×10^6 cells/ml were placed in triplicate in 96-well round bottom microtitre plates containing PHA at concentrations of 1, 10 and 30 μg/ml and con-A at 1, 10 and 100 μg/ml. Lymphocyte cultures were incubated at 37 °C in 5% CO_2. After 72 h, lymphocytes were pulsed with 1 μCi [^{3}H]thymidine for 18 h. Thymidine uptake was determined by precipitation of radioactive lymphocyte DNA on filter paper discs. Discs were placed in standard scintillation fluid (Ultima Gold, Packard, Perkin-Elmer, Waltham, MA, USA) and determination of emissions as counts per minute (cpm) using a scintillation counter. Mitogen stimulation indices were determined as cpm (mitogen stimulated culture)/cpm (unstimulated culture) and an overall MSI obtained as a mean of the triplicate PHA and con-A values. In occasional cultures aberrantly high results were obtained, these presumed to be a result of bacterial contamination, given that bacterial cell walls can be mitogenic in their own right.[10] In these cases such values were disregarded where the values obtained were more than three standard deviations from the mean of the other two values from the triplicate samples.

Cyclosporine from EDTA-anti-coagulated samples was first measured using a standard specific enzyme-multiplied immunoassay method, previously validated by comparison with radioimmunoassay.[11,12] Since this technique gave a low detection limit of 15 ng/ml the more novel quantification technique mass spectroscopy (MS)[13,14] was used to repeat measurements on all samples. While the technique is not widely used, since measuring low levels of cyclosporine is not important in clinical transplant medicine, it has been internally validated to a detection level of 1 ng/ml in the laboratory (Harefield NHS Trust, Harefield, UK) where it was developed (N. Leaver, personal communication).

Statistical evaluation of results involved comparison of repeated measures analyses of variance of MSI between 2 and 0.2% treated dogs. Repeated measures comparison of analyses of variance was also used to compare the differences between circulating cyclosporine values at 1, 3 and 6 months from those at the commencement of the study in both groups.

RESULTS

Animals recruited to study

Breeds, weights and ages and ocular conditions of the animals recruited to the study are shown in Table 1. Mean weights of dogs in 0.2% and 2% treatment groups were 16.4 ± 8.2 kg and 17.6 ± 11.4 kg respectively, these not being statistically different. Mean ages for these groups were 6.7 ± 2.6 years and 6.6 ± 2.5 years, respectively, these similarly not being statistically significantly different. Hematology and serum biochemistry revealed no abnormal findings in any dog (data not shown).

Measurement of blood cyclosporine levels

Using the radioimmunoassay or enzyme-multiplied immunoassay technique (EMIT), no samples contained over 15 ng/ml

Table 1. Dogs investigated in current study

Dog	Group (%)	Breed	Age	Gender	Weight (kg)	Condition
1	2	Shih tzu	5.5	me	7.2	KCS
2	2	Cairn terrier	7.2	fe	7.7	KCS
3	2	German shepherd dog	6.4	mn	34.2	CSK
4	2	Miniature poodle	7.0	me	7.0	KCS
5	2	Standard poodle	12.0	mn	21.0	LPC
6	2	Shih tzu	8.5	me	8.7	KCS
7	2	Boxer cross dog	6.5	fn	28.5	KCS
8	2	German shepherd dog	6.0	fn	32.5	LPC
9	2	German short-haired pointer	3.0	fe	22.3	KCS
10	2	Miniature bull terrier	3.5	fe	6.4	KCS
11	0.2	German shepherd dog	5.5	fn	27.5	CSK
12	0.2	Cross-bred	4.5	fn	7.8	KCS
13	0.2	West Highland white terrier	2.0	me	13.5	KCS
14	0.2	West Highland white terrier	7.9	fn	15.2	KCS
15	0.2	Border Collie	6.0	fn	29.0	CSK
16	0.2	Cocker Spaniel	8.1	fe	20.5	KCS
17	0.2	West Highland white terrier	6.0	me	16.1	KCS
18	0.2	English springer spaniel	11.2	me	24.2	KCS
19	0.2	Lhasa apso	9.8	mn	7.6	KCS
20	0.2	West Highland white terrier	5.7	fn	16.7	KCS

me, male entire; fe, female entire; mn, male neutered; fn, female neutered; CSK, chronic superficial keratitis; KCS, keratoconjunctivitis sicca; LPC, lymphocytic plasmacytic conjuctivitis.

cyclosporine, the lower limit of detection. Positive control samples from dogs treated with oral cyclosporine in the management of anal furunculosis and assayed using the same technique showed levels of between 430 and 620 ng/ml (data not shown), demonstrating that these negative findings were not a consequence of technique failure. Repeat measurement of cyclosporine concentrations using nuclear mass spectroscopy (NMS) showed all samples to contain lower than 1 ng/ml of the drug.

Mitogen stimulation indices

Mitogen stimulation indices obtained in each dog during this study are shown in Fig. 1 and Table 2. Individual animals were not presented for re-examination on dates exactly 1, 3 and 6 months after entry into the trial and thus Fig. 1 shows MSI data points for each animal on the exact day of re-examination. When data were grouped in time intervals of 1, 3 and 6 months, the mean and standard deviation of the MSI data for each treatment group at each examination could be determined, these data being presented as box plots in Fig. 2. Mitogen stimulation index data for each group were not significantly different at any time point. Since mean values of MSI data differed at the first time point, i.e. before treatment had started, the differences in MSI between a given time point and the values at the beginning of the trial for each dog are considered more important than the MSI values themselves, the mean ± standard deviation of these differences being shown graphically in Fig. 3. Comparison by analyses of variance for these data showed no significant difference between changes in MSI between the two different treatment groups at any time-point as demonstrated by the *P* values shown in Fig. 3. Although the difference between change in MSI between treatment groups at

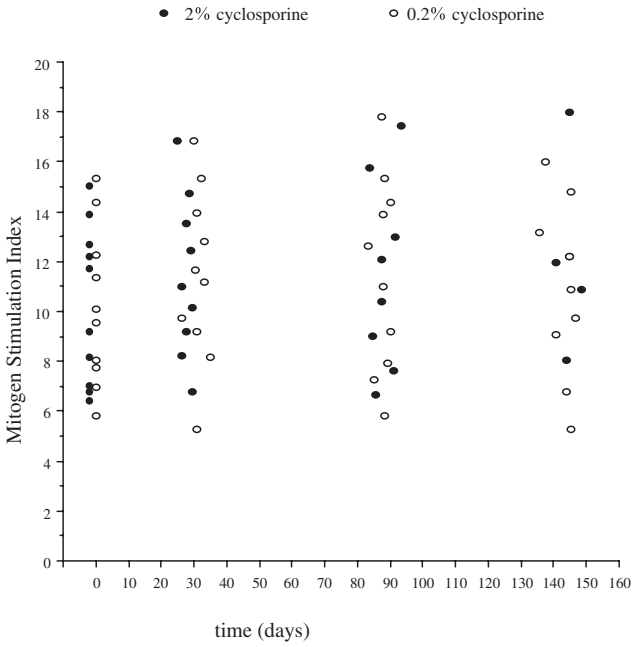

Figure 1. Mitogen stimulation indices for dogs treated with topical 2% and 0.2% cyclosporine over a 6-month period.

6 months of treatment neared significance ($P = 0.0706$) the small number of dogs remaining in the 2% treatment group tends to invalidate this statistical measure.

DISCUSSION

This study contradicts the findings of the two previous reports.[5,6] In this study no circulating cyclosporine was detected in any animal nor were mitogen stimulation

Table 2. Mitogen stimulation indices in dogs over 6 months of treatment

Dog	Concentration (%)	Start of treatment	1 month	3 months	6 months
1	0.2	15.33	16.87	17.81	16.01
2	0.2	14.39	15.33	15.38	14.84
3	0.2	12.31	13.98	14.39	13.17
4	0.2	11.37	12.81	13.89	12.22
5	0.2	10.15	11.68	12.63	10.91
6	0.2	9.56	11.23	11.05	9.79
7	0.2	8.07	9.74	9.25	9.11
8	0.2	7.76	9.2	7.94	6.81
9	0.2	6.99	8.16	7.31	5.32
10	0.2	5.86	5.28	5.86	6.45
11	2	6.99	9.25	7.67	10.91
12	2	7.04	10.19	9.07	12.00
13	2	8.16	11.05	10.42	12.22
14	2	13.89	12.49	12.09	18.04
15	2	9.25	13.58	13.03	11.22
16	2	12.22	14.75	15.78	•
17	2	11.77	16.87	17.5	•
18	2	12.72	•	•	•
19	2	15.06	6.67	•	•
20	2	6.81	•	8.07	•

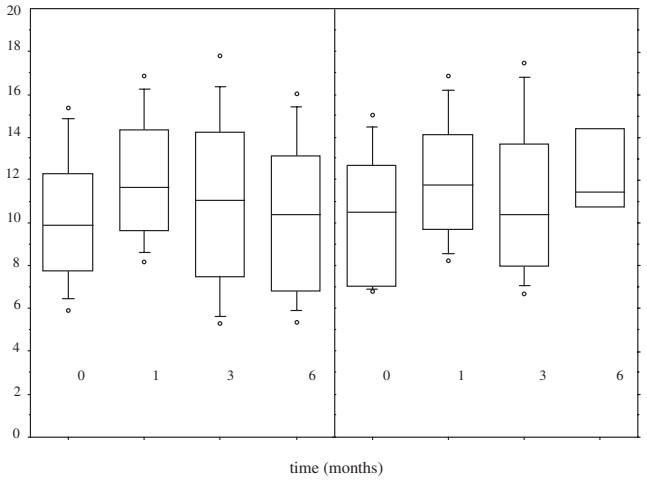

Figure 2. Box plot visualization of data presented in Fig. 1.

indices found to decrease during treatment. In their 1995 publication, Gilger *et al.*[5] did not document measurements of circulating blood levels of cyclosporine but did report MSI data in dogs treated with 2% cyclosporine. Mitogen stimulation index values in that study were reduced from 12.3 ± 8.2 before treatment to 9.5 ± 8.4 at 1 month of treatment and 3.0 ± 8.6 after 3 months. Their 1996 report[6] did not compare pre and intra-treatment MSI values but evaluated MSI at different treatment durations. While linear regression of MSI values in treated dogs against duration of treatment suggested that values decreased with increasing treatment time, the association between time on treatment and MSI value was not statistically significant.

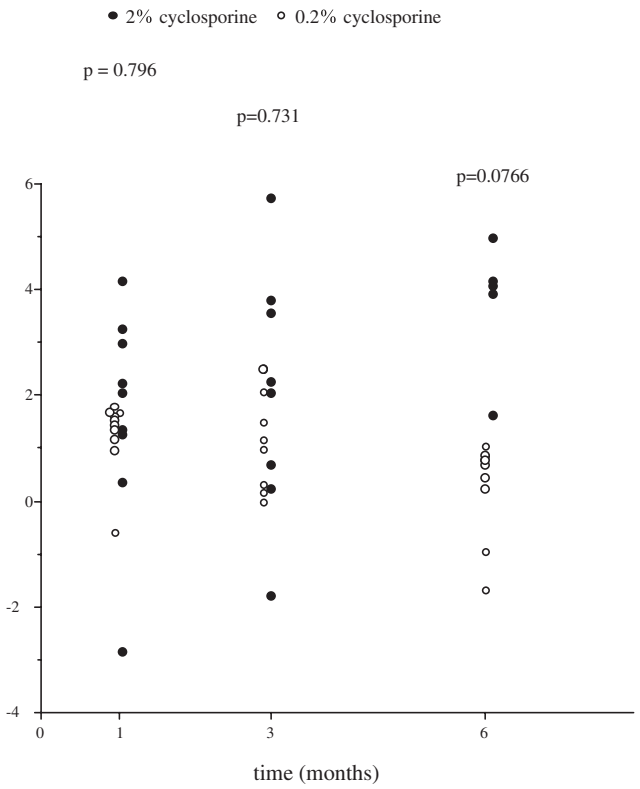

Figure 3. Difference between mitogen stimulation index (MSI) at given time point and initial MSI.

The differences between measurements of circulating cyclosporine levels in the previous work and the present study might be considered difficult to explain. Similar variations in cyclosporine absorption have, however, been reported in previous studies. Some studies have found significant concentrations after topical application: 1% cyclosporine administered to rat eyes five times daily resulted in mean blood levels of 140 ng/ml.[15] Similarly another group have used topical cyclosporine to produce systemic immunosuppression in cats, showing, in this species that a high level of absorption occurs from topically applied drug.[16] Other studies, however, have found no measurable absorption of topically applied drug: one group reported that a 10% cyclosporine ointment given in both eyes of rabbits did not lead to measurable blood levels of cyclosporine,[17] while a more recent study of topically applied cyclosporine in an ointment formulation similarly showed no absorption.[18] Differences between those studies may be explained by variation in concentration of applied drug, formulation of the vehicle used, frequency of application or size of experimental species involved. Differences between the previous reports and the current study cannot, however, be explained by size of animal, frequency of administration nor concentration of drug. Both the previous study and the 2% group of this study used corn oil as the vehicle and thus differences in drop formulation are unlikely to be the cause of the considerable difference between the drug concentrations achieved in the

previous study (26.6 ± 5.7 ng/ml in the six dogs with measurable blood levels out of the ten in that study) and the values in this study which were uniformly below the lowest level measurable by current detection methods (<15 ng/ml for EMIT and <1 ng/ml for MS).

Several areas may be highlighted in which this study could have been improved. First, dogs were allocated to one or other treatment group without attempting to pair similarly sized animals or dogs with similar ocular conditions. Given the inverse correlation between weight and circulating cyclosporine level in the previous study the differences in mean weight of the dogs in the groups in this study could have influenced the results. As it was there was no statistical difference between weight distribution in either group ($P = 0.91$ with unpaired *t*-test). Similarly dogs could have been matched for age, but data showed no significant difference between the two groups with regard to age distribution ($P = 0.78$ with unpaired *t*-test) It might be argued that dogs with the same condition should have been matched since the more vascular corneal lesions in CSK could have promoted drug absorption into the blood. Eight dogs in the 0.2% group and seven in the 2% group had KCS with the remaining dogs being affected with corneal or conjunctival inflammatory disease. No increases in blood levels of cyclosporine were, however, noted in dogs with the vascularized corneal lesions of CSK. Indeed circulating drug levels were below measurable levels by either EMIT or NMS techniques in both groups.

While the study initially aimed to place dogs randomly into the two treatment groups this was not possible for ethical and legal reasons. The cascade system in the United Kingdom requires that, where a veterinary licensed product exists, this must be used to the exclusion of all others. The only exclusion from this rule is where the licensed product is considered unacceptable, usually because of a reaction against the preparation but also where the product is not efficacious. Thus here, the animals in which the 2% nonlicensed product was used were all dogs in which the licensed product was not having the desired effect of increasing tear production. In some cases this was likely to be because of owner noncompliance: in six of the 10 animals where 0.2% cyclosporine had not previously shown a lacrimogenic effect, owner noncompliance may have been an important factor in drug failure: owners found difficulty in applying ointment and stated that a drop formulation was far easier to use. In the other four animals the reason for lack of effect of 0.2% ointment was unclear. It has previously been demonstrated that a proportion of animals failing to show increased tearing with 0.2% cyclosporine will show an effect with 2% cyclosporine.[19] Given the fact that only dogs where 0.2% ointment was ineffective were assigned to the 2% drop formulation group, it was not possible randomly to place the dogs in one or other group. This is not considered to have markedly affected the results of the trial. The loss of cases towards the end of the study through owner noncompliance was disappointing; when asked the reason for failure to attend the majority of nonattenders explained that the dog's ocular comfort was acceptable and that geographic distance was generally their reason for failing to attend the last clinical appointment.

A potentially important technical shortcoming was that MSI determinations were made for each dog on the day the blood was taken. This may have resulted in an increased natural variation in data which could have been reduced if lymphocytes had been cryopreserved and MSI data determined for all samples in one large batch measurement. Variations in MSI values between dogs and within tests are to be expected as this test determines biological function of lymphocytes and not merely a drug concentration or hormone level.

The hypothesis of this study was that while the group of animals treated with the 2% oil-based formulation would show changes similar to those in the two previous reports,[5,6] the 0.2% ointment based treatment group would not show drug absorption to the same degree. It was considered that the 10-fold lower concentration and the ointment formulation would significantly reduce systemic drug absorption. As a consequence of this it was expected that lower circulating drug concentrations would be detected in serum and less pronounced functional changes would be detected in lymphocytes. Thus it was surprising to be unable to repeat the findings of the two previous reports with either a 0.2% ointment formulation or a 2% oil-based preparation. Indeed while there was no change in MSI values during treatment with 0.2% cyclosporine in ointment, dogs treated with 2% cyclosporine in the corn oil drop formulation showed an increase in MSI at 6 months of treatment, this nearing statistical significance. This result was, however, based on only five dogs completing the 6-month treatment course. The other five dogs in this treatment group were not brought for final re-examination by their owners since the animals were in good health with the treatment given having ameliorated their ocular disease fully. As shown in Figs 1,2, the variation in MSI values was not significantly different for this last evaluation at 6 months, compared with previous measurements, so the loss of these animals form the study is not considered to have changed the findings markedly.

While no ready explanation for the difference between this study and the two previous reports is obvious, the results of this study would appear consistent with clinical findings that dogs using topical cyclosporine do not appear subject to any signs of systemic immunosuppression. Thus the findings here that topical cyclosporine at both 0.2% and 2% has no discernible effect on proliferative activity of circulating lymphocytes is not particularly surprising but may be valuable in demonstrating the safety of these drugs as currently topically formulated.

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