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Preliminary investigations into pharmacodynamic monitoring of cyclosporine in cats

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Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Research
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Existing pharmacokinetic monitoring tools for cyclosporine fail to correlate with clinical response. In dogs, pharmacodynamic monitoring of nuclear factor of activated T cell (NFAT) regulated cytokines is thought to provide a better overall evaluation of the immune response to cyclosporine than blood levels; however, such monitoring tools are not available in cats. In this study, we designed and optimized a protocol for maximal T lymphocyte stimulation in cats. This is the first step in the development of a pharmacodynamic monitoring tool for cyclosporine in cats based on expression of NFAT-regulated cytokines. We also confirmed that cyclosporine has anti-lymphocytic properties in cats, and we were the first to document induction of apoptosis by cyclosporine in cats. Differences in individual patient response to cyclosporine may be influenced by apoptotic response of lymphocytes to cyclosporine. Additional studies are required to optimize and validate polymerase chain reaction monitoring of NFAT-regulated cytokines for cyclosporine-mediated immunosuppression.

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DEDICATION

I would like to dedicate this research to my parents, Simon Henri Cridge and Lisa Cridge, for their unwavering support and trust. They have always provided me with the motivation to overcome difficulties throughout my professional pursuits. Without them, I would not be the person I am today.

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CHAPTER I

LITERATURE REVIEW

Background

Cyclosporine is a fat-soluble, hydrophobic polypeptide derived from the fungus *Beauvaria nivea*[1,2]. Cyclosporine was discovered in 1971 as part of a screening program of fungal metabolites for immunosuppressive activity, and was subsequently purified, with initial studies published in 1976[3,4]. Cyclosporine was welcomed by the research community and was investigated as a potential drug in the prevention of organ transplant rejection. Studies in rats documented that cyclosporine could prolong heart allograft acceptance, and further studies in pigs and dogs evaluated its use in orthotopic heart grafts and renal allografts, respectively[3,5,6]. Cyclosporine was approved by the Food and Drug Administration (FDA) in 1983 and has subsequently become a cornerstone of immunosuppression in transplantation medicine[7,8]. Although the vast majority of early work occurred in humans, dogs and laboratory animals, Latimer and colleagues evaluated the effects of cyclosporine in cats in 1986[9]. In this study, oral cyclosporine administration resulted in moderate to marked variation in trough cyclosporine concentrations between cats, and there was no correlation between serum cyclosporine concentration and lymphocyte transformation[9]. This highlighted the significance of cat-to-cat variation in response to cyclosporine. In 2011, the FDA approved a modified cyclosporine oral suspension for the management of feline allergic dermatitis. Despite being approved only for use in hypersensitivity dermatitis, similar to in dogs, this feline cyclosporine suspension is finding

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off-label use for a large number of dermatological and systemic inflammatory conditions in cats. Unfortunately, optimal dosing protocols for achieving immunosuppression with cyclosporine in cats remain unclear and methods that objectively monitor effectiveness of immunosuppression have not been established. Ideally, effectiveness of immunosuppression should be individually monitored to ensure that the patient is truly immunosuppressed, and that the cyclosporine is effectively treating the condition.

Mechanisms of Action of Cyclosporine

Immunosuppressive Effects

In the absence of cyclosporine, antigen in conjunction with major histocompatibility complex binds to the T-cell receptor (TCR) on the surface of a T lymphocyte. Binding to the TCR results in an increase in intracellular calcium. This increase in intracellular calcium causes activation of calcineurin. Calcineurin is an intracellular protein phosphatase that activates gene transcription factors via dephosphorylation of the cytoplasmic form of nuclear factor of activated T cells (NFAT)[10]. Once dephosphorylated, the activated NFAT migrates into the nucleus, where it upregulates transcription of genes coding for many cytokines, including interleukin-2 (IL-2), interleukin-4 (I-4), interleukin-5 (IL-5), interleukin-13 (IL-13), granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) [11] Cyclosporine acts as a calcineurin inhibitor. Cyclosporine passes into the cytoplasm of the T lymphocyte and binds cyclophilin A, a chaperone protein. The cyclosporine-cyclophilin A complex has a high affinity for calcineurin, inhibiting its activation[10]. This ultimately prevents upregulation of NFAT-regulated genes and reduces cytokine production, in particular IL-2[1]. In T-lymphocytes, IL-2 binds to the IL-2 receptor, leading to down-stream signal processing,

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activation and proliferation of T lymphocytes[12]. Thus, blocking IL-2 production reduces lymphocyte proliferation.

Anti-inflammatory Effects

Cyclosporine has two primary mechanisms of action in inflammatory dermatological disease. Firstly, it decreases cytokine production by inflammatory infiltrates and, secondly, it inhibits the epidermal cytokine network[21]. In addition to its effects on lymphocytes, cyclosporine has also been shown to have anti-inflammatory effects on basophils, mast cells, macrophages, eosinophils, neutrophils, keratinocytes, and endothelial cells[16,21–35]. These mechanisms contribute to its anti-inflammatory properties

Anti-proliferative Effects

Cyclosporine has been shown to inhibit proliferation of keratinocytes at the G_0 and G_1 phases of the cell cycle; however, it is not known if these effects are clinically relevant, as the doses required may not be achievable *in vivo* [1,36]

Other Effects

Cyclosporine is extensively utilized in veterinary ophthalmology, primarily for treatment of immune-mediated keratoconjunctivitis sicca (KCS) in dogs, although its safety and efficacy have not been prospectively evaluated in cats with KCS[37]. A 2009 retrospective study did however document the successful use of a topical 1.5% cyclosporine solution in the management of proliferative feline eosinophilic keratitis, and a subsequent article also documented successful use of a topical cyclosporine solution in cats that failed to respond to topical corticosteroids[38,39]. Cyclosporine's lacrimometic effects are mediated via anti-prolactin receptor activity[14].

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Cyclosporine has also been shown to have anti-parasitic properties; however, it is unlikely to be used clinically to control parasitic infections[1]

Pharmacokinetics

Absorption

Following oral administration, cyclosporine is absorbed across the intestinal epithelium via passive diffusion. As cyclosporine is a P-glycoprotein substrate, its intestinal absorption is limited via efflux pumps[40]. The licensed formulation for cyclosporine in cats is a microemulsion, and other non-microemulsified formulations such as Sandimmune (Novartis Pharmaceuticals) are no longer routinely used[41]. Sandimmune has poor and highly variable oral bioavailability in humans, and is therefore not recommended in veterinary species[2]. The estimated oral bioavailability of the microemulsion formulation of cyclosporine in cats is 29% after 7 days of administration, and 25% after 14 days of administration[42]. Oral bioavailability does not appear to be affected by food, unlike in dogs[43,44]. In addition, ocular, transdermal and subcutaneous administration have been studied in cats. Ocular and transdermal administration of cyclosporine is reported to result in inconsistent absorption, whereas subcutaneous administration appears to be efficacious in a study evaluating alternate day dosing in the management of feline allergic dermatitis [45–47].

Distribution

Following oral absorption, cyclosporine is distributed widely, and accumulates in the skin and adipose tissue[48,49]. The volume of distribution is reported to be 1.71 L/kg following intravenous administration, and 5.95 L/kg following oral administration[42]. Peak blood

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concentrations occur within 1-2 hours of oral administration of cyclosporine in cats, and peak concentrations are highly variable amongst cats, with a coefficient of variation of 31%[42].

Metabolism

The major sites of cyclosporine metabolism are the liver and, to a lesser extent, the small intestines and kidneys[50]. Cyclosporine is a known substrate of cytochrome P-450 3A in humans, and feline responses to ketoconazole, itraconazole and clarithromycin suggest that this may also be true in cats[51–53]. Hepatic metabolism of cyclosporine is primarily via N-demethylation and hydroxylation of amino acid residues[54]. M-21 is the primary N-demethylated cyclosporine metabolite, whereas M-1 and M-17 are the primary hydroxy metabolites of cyclosporine[55]. The composition of cyclosporine metabolites is important, as metabolites such as M-17 have *in vitro* immunosuppressive activities, and their concentration can exceed that of the parent drug[56]. In cats, the concentrations of cyclosporine metabolites differ from in dogs, with cats having approximately equal concentrations of M-17, M-1 and M-21[55]. In contrast, dogs have lower levels of M-17 relative to other metabolites[55].

Cyclosporine metabolism can be influenced by drugs that induce or inhibit cytochrome P450 enzyme systems. Although a large amount of research has been performed in this area, little research has been performed specific to cats, and many potential drug interactions are inferred from other species. In 2007, Shah et al. investigated cytochrome P450-mediated drug metabolism in cats and noted that sexual differences were present in the activity levels of cytochrome P450 (CYP)2D and CYP3A, with CYP2D being higher in females and CYP3A being higher in male cats[57]. This research suggests that different doses of CYP2D and CYP3A drugs may be required in male and female cats. Interestingly, the rate of elimination of CYP3A substrates in female cats was similar to that in people, whereas the rate in male cats was more

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similar to dogs[57]. Ketoconazole is an azole antifungal medication which inhibits CYP3A[58]. Concurrent administration of ketoconazole with cyclosporine has been shown to inhibit metabolism of cyclosporine and increase blood cyclosporine levels; this combination may be used for clinical benefit[51,59–63]. In cats, ketoconazole administration results in a 1.8-2.2 fold increase in blood cyclosporine levels, and an approximately 60% reduction in systemic clearance rate[51]. A 2009 study evaluated the effect of ketoconazole on feline hepatic microsomes and CYP3A activities, via evaluation of midazolam 1' and 4-hydroxylation. In this study, it was noted that ketoconazole was effective in inhibiting feline hepatic CYP3A activity, albeit to a much lesser extent than occurred in either humans and dogs[64]. In the same study, cimetidine and erythromycin did not affect CYP3A activity in cats[64]. Despite the differences between cats and other species with regard to effect of azole drugs on CYP3A activity, both itraconazole and ketoconazole have been shown to result in significant increases in cyclosporine blood levels in cats[51,52]. In addition, clarithromycin was recently studied as a potential agent to reduce both the dose and frequency of cyclosporine in feline transplant patients. In the study, clarithromycin administration resulted in a 65% reduction in the dose of cyclosporine required to reach a pre-determined trough cyclosporine level; it also allowed for once daily administration of cyclosporine due to the increased half-life of cyclosporine when combined with clarithromycin[53].

Excretion

Cyclosporine has an elimination half-life of 8 hours in cats, and its metabolites are excreted through the biliary system, with minimal renal excretion[41,42,50,65].

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Licensed Indications

Cyclosporine is the only FDA-approved immunosuppressive agent in cats. It is licensed as a microemulsion formulation (Atopica®) for the management of feline allergic dermatitis. Atopica® is approved for cats of at least 6 months of age and weighing at least 1.4kg[66]. According to the manufacturer, Atopica® should be used with caution in patients with diabetes mellitus or renal insufficiency[66]. The recommended dose is 7mg/kg orally every 24 hours, until resolution of clinical signs, followed by a taper[50].

Other Dermatological Uses

A retrospective case study, published in 2012, revealed that modified cyclosporine was effective in the management of pemphigus foliaceus in cats, and had a glucocorticoid-sparing effect[67]. Additional literature on cyclosporine in feline dermatology is limited to case reports or case series and, as such, caution should be advised when applying the data to larger populations of animals. Case reports and case series document the use of cyclosporine in feline lymphocytic mural folliculitis, feline urticaria pigmentosa, idiopathic facial dermatitis, plasma cell pododermatitis and pseudopelade[68–72]. Over time, it is likely that cyclosporine usage in feline dermatology will increase due to its reported clinical efficacy and favorable toxicity profile.

Renal Transplantation

Preliminary studies into renal allografts in dogs and cats revealed that a combination of prednisolone and cyclosporine could be effective in the management of renal transplants, given appropriate patient selection[73]. These early studies also stressed the importance of pharmacologic and immunologic monitoring of renal transplantations, and subsequent individual dose adjustments in therapy[73]. Following these initial reports a case study was published that

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documented survival of a 6-year Persian cat for greater than 18 months with a renal transplant from an unrelated donor, further prompting additional research into the use of prednisolone and cyclosporine for immunosuppression in transplant recipients [74]. Over time, the number of patients receiving a combination of prednisolone and cyclosporine increased, and a large retrospective study on feline renal transplants was published in 2008. In this paper, 77.5% of patients with a renal transplant survived to hospital discharge and the median survival time was almost 2 years[75]. In the study, however, 37% of cases developed an infection post-transplantation. The high prevalence of infection in renal transplant patients despite attempts to monitor trough cyclosporine levels indicates the need for further research into alternative monitoring tools for cyclosporine in cats.

Systemic Inflammatory Diseases

Cyclosporine has been used off-label in a number of systemic inflammatory conditions, including asthma, hematological disorders, inflammatory bowel disease and chronic progressive polyarthritis. Dosages used in feline systemic inflammatory diseases vary widely (Table 1.1).

Cyclosporine has been studied extensively in feline asthma. *In vitro* airway hyper-responsiveness and eosinophil yield were reduced in bronchoalveolar lavage fluid (BAL) following treatment with cyclosporine in chronically antigen-challenged cats[76]. Clinicians have subsequently utilized cyclosporine in cases where glucocorticoids were contraindicated. One such case report documented the use of cyclosporine in a cat with asthma which could not receive steroid medication due to concurrent diabetes mellitus and congestive heart failure[77]. Cyclosporine is a promising medication in the management of feline asthma in cases where concurrent disease limits steroid use. Cyclosporine has also been documented in the management of feline pure red cell aplasia[78–80], immune-mediated thrombocytopenia[81], idiopathic

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inflammatory bowel disease[82,83] and chronic progressive polyarthritis[84]. Cyclosporine is typically considered in these conditions when glucocorticoids alone are insufficient to induce disease remission or when steroids are otherwise contra-indicated. Anecdotally, some clinicians are also using cyclosporine as a second immunosuppressive agent in patients that cannot tolerate glucocorticoid side effects, thus allowing for a more rapid taper in glucocorticoid dose

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Table 1.1 Starting doses of cyclosporine in feline systemic inflammatory diseases

Disease	Paper	Cyclosporine Dose	Dose Frequency
Feline Asthma	Nafe and Leach, 2015	4mg/kg	PO q 12h
Pure Red Cell Aplasia	Stokol and Blue, 1999	20mg/kg	PO q 24h
Immune-Mediated Thrombocytopenia	Garon et al., 1999	5mg/kg	PO q 12h
Inflammatory Bowel Disease	Jergens, 2012	5mg/kg	PO q 12-24h
Chronic Progressive Polyarthritis	Oohashi, 2009	7.35mg/kg	PO q 24h

Starting doses of cyclosporine for various inflammatory diseases in cats described in the literature. There is wide variation in dose and dose frequency utilized, corresponding with a lack a consensus on the starting dose of cyclosporine for systemic immunosuppression in cats. Additional research is needed in this area.

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Recommended Pre-treatment Examination

Due to cyclosporine's unpredictable immunosuppressive properties, it is critical that veterinarians rule out infectious disease prior to administration. It has been recommended that veterinarians evaluate a through history and physical examination, in addition to performing bloodwork (complete blood count, serum chemistry, urinalysis, feline leukemia virus and feline immunodeficiency virus serology) prior to treatment[41]. Toxoplasmosis remains an important concern in cats being treated with cyclosporine. Some authors report that being seronegative increases the risk of developing clinical toxoplasmosis (if exposed) and therefore believe that seronegative patients require more careful management[50,85]. In contrast, some authors report that cyclosporine therapy re-activates latent infections and therefore believe that seropositive patients are at a greater risk of clinical toxoplasmosis[86]

Drug Interactions

Cyclosporine has proven drug interactions with ketoconazole, itraconazole and clarithromycin in cats[51–53]. However, multiple other drug interactions are possible due to drug effects on the cytochrome P450 enzyme system. Drug interactions in other species have been noted with the following frequently used drugs in cats: angiotensin-converting enzyme inhibitors, allopurinol, aminoglycosides, cephalosporins, chloramphenicol, digoxin, diltiazem, fluconazole, glucocorticoids, non-steroidal anti-inflammatory drugs, phenobarbitone, rifampin and trimethoprim sulfa[2]. Additional studies are required to determine whether these drugs represent clinically important drug interactions in feline patients.

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Complications of Cyclosporine Therapy

A clinical safety study for modified cyclosporine in cats documented multiple adverse effects. In the study, cats were assessed via a six-week field study at 7mg/kg/day as a single daily dose, followed by a twelve-week open label dose-tapering field study. The following adverse effects were noted: vomiting (35.1%), weight loss (20.5%), diarrhea (15.1%), anorexia (14.1%), lethargy (13.6%), hypersalivation (11.2%), behavioral disorders (8.8%), ocular discharge (6.8%), sneezing (5.4%), gingival hyperplasia (4.4%) and polydipsia (2.9%). Other less common adverse effects included bacterial dermatitis, hepatic lipidosis, small cell lymphoma, constipation, toxoplasmosis, muscle wasting, ataxia, convulsions, polyuria, urinary tract infection, inappropriate urination or defection, seborrhea, otitis externa, papilloma development, leukotrichia, hypertrichosis, CBC abnormalities and increased creatinine, blood urea nitrogen and alanine aminotransferase[66]. In other studies, gastrointestinal signs were the most prominent adverse effects[44,87–92]. Additional studies have reported the following rare adverse effects: acute bullous keratopathy and hemolytic uremic syndrome[93,94].

Rarely, cyclosporine has been associated with the development of malignant neoplasia, which may be associated with inhibition of cytotoxic T-cell mediated anti-tumor immune surveillance[95]. In humans, cyclosporine is associated with a two-fold increase in the risk of malignancy, with cyclosporine-treated organ transplant patients being at a twenty-eight fold increased prevalence of lymphoma[96]. Lymphoma has also been reported in a case report of a dog being treated with cyclosporine[97]. Multiple retrospective papers have investigated the incidence of post-transplantation malignant neoplasia in cats treated with cyclosporine, with an incidence rate of 9.5-24%[98,99]. The incidence rate of malignant neoplasia appears lower in patients treated with cyclosporine for dermatological or immune-mediated disease conditions[50].

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Opportunistic infections, in particular toxoplasmosis, are another significant concern in patients undergoing cyclosporine therapy. In 1999, the first potential link between cyclosporine and development of toxoplasmosis was documented. In two of the three cases tachyzoites were identified in the organs, but not in the allografts, suggestive of reactivation of a latent infection following initiation of cyclosporine therapy[100]. However, a 2004 case report documented a newly acquired fatal toxoplasmosis infection in a cat receiving cyclosporine at the licensed dose[101]. This case report highlights the potential of excessive immunosuppression in individual patients on doses of cyclosporine traditionally indicated for dermatological conditions. This prompts further investigation into appropriate therapeutic drug monitoring techniques in cats. With regard to toxoplasmosis, it is not always clear whether the patient had reactivation of a chronic infection or developed a new infection with toxoplasmosis and, as such, serology for toxoplasmosis is recommended prior to therapy[100]. Lappin et al., inoculated cats with *Toxoplasma gondii* to evaluate the effect of cyclosporine on infection status. In this study, it was demonstrated that administration of oral cyclosporine increased the severity of *T. gondii* infection in naïve animals, but not in those previously exposed to *T. gondii*[85]. Cats on cyclosporine should be kept indoors and should not be fed raw meat so as to minimize exposure to toxoplasmosis. Animals that are seronegative prior to therapy may be at an increased risk of clinical toxoplasmosis[50,85]. Cyclosporine has also been documented to reactivate feline herpesvirus-1 infection; however, most clinical signs are mild and severe clinical manifestations are rare[102].

Monitoring of Cyclosporine Therapy

Variability in individual response to cyclosporine has been documented in both dogs and cats[2,50]. Potential explanations include differences in bioavailability, metabolism and T-cell responses such as cytokine expression, lymphocyte proliferation, and/or apoptosis. Differences

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in individual patient response can lead to excess immunosuppression and development of fatal infections, or to an absence of immunosuppression and undertreatment. Therapeutic drug monitoring is therefore recommended in patients receiving cyclosporine[2,50]. Therapeutic drug monitoring has been extensively reviewed in dogs[2]; however, cyclosporine blood levels are the only commercially available tool for therapeutic monitoring in cats. Additional research is therefore needed to develop additional tools for therapeutic monitoring of cyclosporine in cats

Pharmacokinetic Monitoring in Cats

Trough Cyclosporine Concentrations

Recommendations for monitoring of cyclosporine therapy in cats include measurement of trough cyclosporine levels in whole blood[103]. Whole blood is the sample of choice, as cyclosporine concentrates in red blood cells[2]. A target twelve hour trough cyclosporine concentration of 300-500ng/mL is recommended; however, trough levels are highly variable and have a poor correlation with clinical efficacy[9,42,103] This poor correlation has led to calls to re-consider therapeutic drug monitoring in cats[42]

Peak Cyclosporine Concentrations

Mehl et al., evaluated whole blood concentrations of cyclosporine following oral administration in six healthy cats. In this study, cyclosporine concentrations two hours after cyclosporine dosing correlated more closely with area-under-the-curve results than 12-hour post administration concentrations. This suggests that peak whole blood cyclosporine levels may be a better tool to evaluate cyclosporine therapy in cats[42]. However, therapeutic peak target concentrations have not yet been established, thus limiting their clinical utility[2,42].

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Area Under the Concentration-Time Curve (AUC) Monitoring

AUC monitoring is more highly correlated with clinical outcomes than trough cyclosporine levels in humans[104], and AUC was used as the gold standard in a recent feline study comparing peak and trough cyclosporine levels[42]. Additional research is needed to determine if AUC monitoring corresponds with clinical immunosuppression, and how it compares to peak and trough cyclosporine monitoring. However, clinical application may be limited by the number of blood samples required for AUC determination.

Pharmacodynamic Monitoring in Cats

Pharmacodynamic assays investigate cyclosporine's effect on lymphocytes. Pharmacodynamic monitoring of cyclosporine may include proliferation assays, assessment of cytokine suppression or calcineurin inhibition assays. Pharmacodynamic monitoring is routinely used in dogs; however, to date there are no studies on pharmacodynamic assays for cyclosporine in cats. Proliferation assays have been utilized for many years and show a high correlation with cyclosporine blood levels. Commonly utilized methodologies include measurement of the proliferation responses of stimulated lymphocytes under the influence of cyclosporine. Proliferation assays were first evaluated by Hibbins et al., who activated lymphocytes with phytohaemagglutinin (PHA), and then incubated them with multiple drugs, including cyclosporine. The gradient of inhibition then indicated the response to cyclosporine[105]. One of the most commonly explored pharmacodynamic monitoring tools explored in dogs is inhibition of lymphocyte cytokine suppression by cyclosporine. Cyclosporine is known to suppress production of many NFAT regulated cytokines, including IL-2. Measuring T cell IL-2 and IFN-γ suppression by cyclosporine using flow cytometric or PCR-based methods is commonly utilized in dogs to determine the level of immunosuppression from cyclosporine[2,106,107].

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Calcineurin inhibition assays measure the direct effect of cyclosporine; however, they are rarely performed due to their technically challenging nature. There are two different methodologies for calcineurin inhibition assays. The first technique involves measurement of inhibition of target enzyme activity, and the second involves measurement of immunological responses[108].

Monitoring Conclusions

Therapeutic drug monitoring is indicated due to marked individual variations in response to oral cyclosporine. Current therapeutic monitoring tools in cats are limited to pharmacokinetic monitoring, and do not appear to correlate well with clinical efficacy. Therefore, additional studies are required to allow for effective monitoring of cyclosporine therapy in cats. Pharmacodynamic monitoring offers promise in this area

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CHAPTER II

EFFECTS OF CYCLOSPORINE ON FELINE LYMPHOCYTES ACTIVATED IN VITRO[1]

Introduction

Cyclosporine A (CsA) is a calcineurin inhibitor licensed for the management of atopic dermatitis in cats. Due to its inhibition of T-lymphocyte function[1–3] and favorable toxicity profile, cyclosporine is also being used with increasing frequency in cats to prevent renal transplant rejection[4,5], and in the management of inflammatory and immune-mediated diseases such as inflammatory bowel disease[6], immune-mediated hemolytic anemia[7,8], immune-mediated thrombocytopenia[9,10], chronic progressive polyarthritis[11,12] and pure red cell aplasia[13], amongst others.

Variability in individual responses to cyclosporine has been documented in both dogs[3] and cats[14]. Factors that help explain dissimilarity in patient responses include differences in bioavailability, metabolism, and T cell responses such as cytokine expression, lymphocyte proliferation and/or apoptosis.

Previous research has shown that cyclosporine decreases the expression of nuclear factor of activated T cell (NFAT)-regulated cytokines including IL-2, IFN-γ and TNF-α in people[15], IL-2 and IFN-γ in dogs[16], and IL-2, IL-4, IFN-γ and TNF-α in cats[1]. Decreased expression of these cytokines results in decreased T-cell function and proliferation, thereby leading to

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immunosuppression. Although the effects of CsA on cytokine expression have been evaluated[1], the direct effects of CsA on lymphocyte proliferation have not been evaluated in cats. Human literature documents that cyclosporine also induces apoptosis of lymphoid cells, which is suspected to contribute in part to its immunosuppressive properties[17]. The effects of CsA on lymphocyte apoptosis have also not yet been evaluated in cats. Thus, further studies are warranted to investigate the effects of CsA on feline lymphocyte proliferation and apoptosis

Cyclosporine-mediated immunosuppression can have significant unintended clinical consequences, including the development of secondary infections such as toxoplasmosis and feline herpesvirus-1[14]. A case report published in 2004 documented that a cat receiving the licensed dose of cyclosporine developed a fatal toxoplasmosis infection[18]. The authors suspected that the cat had a marked reduction in T-lymphocyte function induced by cyclosporine, although it was unclear why this individual cat had such significant immunosuppression relative to other cats receiving the same drug dosage. Case reports such as this prompt further investigation into the immunosuppressive mechanism of cyclosporine in cats, and into individual responses to the drug. Given concerns regarding the potential for the development of clinical toxoplasmosis in cats treated with cyclosporine, Lappin et al. investigated the effects of cyclosporine on both naïve and *Toxoplasma* seropositive individuals that were subsequently infected with *T. gondii*. In the study, it was noted that cyclosporine therapy worsened the severity of infection in naïve cats, but not in those cats that had been previously exposed to *T. gondii.* This experimental study suggests that naïve cats may be at greater risk of clinical toxoplasmosis during cyclosporine therapy[19]. In contrast, extensive clinical experience with feline renal transplantation patients receiving cyclosporine as part of their associated immunosuppressive therapy suggests that pre-

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treatment positive *Toxoplasma* serology actually increases the risk of subsequent clinical toxoplasmosis, consistent with drug-associated recrudescent of occult disease[20]

Therapeutic drug monitoring (TDM) is recommended in patients receiving cyclosporine[3,14], with two methods commonly utilized. One common method is pharmacokinetic monitoring, via measurement of peak and/or trough cyclosporine blood concentrations. However, there are no clear guidelines on therapeutic targets[3], and cyclosporine blood concentrations do not appear to correlate with clinical efficacy in cats[4]. It has therefore been suggested that TDM strategies for cyclosporine in cats should be re-evaluated[21]. Pharmacodynamic monitoring is available in humans and dogs for TDM of cyclosporine, and is considered to supplement information provided by assessment of cyclosporine blood concentrations[3]. Pharmacodynamic assays for TDM of cyclosporine have not yet been established in cats.

The purposes of this study were, firstly, to design and optimize a suitable activation protocol for T cells in cats for subsequent pharmacodynamic assessment of cyclosporine and, secondly to determine whether effects on lymphocyte proliferation and/or apoptosis contribute to the immunosuppressive action of cyclosporine in cats.

Materials and Methods

Experimental Animals

This project involved 6 healthy client-owned domestic shorthair cats. Six to eight animals have been suggested as sufficient population size to evaluate the pharmacokinetics of a drug[22]. Two cats were male neutered, and 4 cats were female spayed. The average age at the time of enrollment was 8 years and 4 months. Prior to inclusion in the study, each cat received a physical examination, and a complete blood count and serum biochemistry profile were evaluated, with

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no significant abnormalities noted. The cats were not on any medications at the time of inclusion in the study. Informed owner consent was obtained before sample collection, and restraint and blood collection protocols were approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC).

Blood Sampling and Peripheral Blood Mononuclear Cell (PBMC) Isolation

Heparinized peripheral blood was obtained via jugular puncture. Initially, 5 mL of whole blood from each cat was added to 45mL of phosphate buffered saline (PBS). The sample was then centrifuged at 500 g for 7-10 minutes to wash the cells. Next, cell suspensions were layered on Histopaque®-1077 (Sigma Aldrich, St Louis, MO, USA), and centrifuged at 500 *g* for 30 minutes to obtain enriched peripheral blood mononuclear cells (PBMCs). Following centrifugation, PBMCs were collected from the interface and were washed three times with PBS at 500 *g* for 7-10 minutes. Residual erythrocytes were removed from the PBMCs with lysis buffer (BD Biosciences, San Jose, CA, USA) for 5 minutes with gentle agitation. Then, PBMCs were counted and assessed for viability by using a hemocytometer and trypan blue exclusion dye. The cells were then used immediately for *in vitro* stimulation studies.

Lymphocyte Stimulation Protocols and Cell Proliferation Assessment

Enriched PBMCs were cultured *in vitro* in medium [RPMI-1640 medium (Sigma Aldrich, St Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum (Sigma Aldrich, St Louis, MO), 100U/mL of penicillin and streptomycin (Sigma Aldrich, St Louis, MO), and 0.25µg/mL amphotericin B (Sigma Aldrich, St. Louis, MO)]. Preliminary experiments revealed that ionomycin (0.6-1.0 µM) and phorbol myristate acetate (10.0-15.0 µg/mL) at varying concentrations failed to result in stimulation of feline lymphocytes (data not shown).

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Therefore, 5µg/mL concanavalin A (ConA) at 5 CO_2 % was selected for the stimulation of lymphocytes based on previous studies [1]. Cell suspensions were transferred to 96-well plates (Evergreen Scientific, Rancho Dominguez, CA), and stimulator and inhibitors were added to cell suspensions.

Four groups of cell culture were analyzed as follows:

i) Cells only (negative control)

ii) Cells + 5µg/mL ConA

iii) Cells + 500 ng/mL CsA

iv) Cells + 5µg/mL ConA + 500 ng/mL CsA

All treatments were incubated at 37.0°C and 5% CO_2. Cell suspension viable cell counts were analyzed daily for 6 days using 0.4% trypan blue exclusion dye (Sigma Aldrich, St. Louis, MO) and a Neubauer hemocytometer chamber (Webber Scientific, Hamilton, NJ). Cells that did not have intracellular staining with trypan blue stain were recorded as "alive", and cells that had intracellular staining were recorded as "necrotic".

Apoptosis Assay

An apoptosis assay was applied as described previously, with minor modifications[23], to PBMCs from a randomly selected cat. Cell suspensions were harvested and washed in PBS by centrifugation at 500 *g* for 7 minutes. Apoptosis was assessed by using an Annexin V-FITC Apoptosis Kit according to manufacturer's instructions (BioVision, Inc., Mountain View, CA). Briefly, after washing, the cells were resuspended in 1x Binding Buffer and incubated with Annexin V-FITC and propidium iodide for 5 minutes at room temperatures in the dark to detect

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early and late apoptotic changes. The samples were then analyzed by NovoCyte Flow Cytometry using two-color analyses with Dot Plot Quadrant Statistics.

Enriched PBMCs were cultured *in vitro*, in the same manner as for the lymphocyte proliferation study noted above. Four groups of cell culture were then analyzed as follows:

i) Cells only (negative control)

ii) Cells + 5µg/mL ConA

iii) Cells + 500 ng/mL CsA

iv) Cells + 5µg/mL ConA + 500 ng/mL CsA

The apoptosis assay was performed on day 1 and day 5. Each treatment was performed and analyzed in triplicate, with the exception of the negative control group which was not repeated.

Statistical Analysis

Live cell counts were log10 transformed prior to analysis. A linear mixed model using PROC MIXED in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC) was fit for live cells, in a model that included day, treatment and their interaction as fixed effects. Cat identity was included as a random effect with variance components covariance structure. In the case of the significant interaction term, differences in least squares means between each pair of days for each treatment group were calculated and between ConA and ConA-activated PBMCs in the presence of CsA treatment groups for each day using an LSMESTIMATE statement.

Linear models with PROC MIXED in SAS for Windows 9.4 were used for percentage of live cells, early apoptotic cells, late apoptotic cells, and necrotic cells. Initial models for late apoptosis and necrosis did not meet the assumptions of normality and homoscedastiticity; therefore, the data for these outcomes was log10 transformed. Fixed effects for each outcome

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(live cells, early apoptotic cells, log10 late apoptotic cells, and log10 necrotic cells) were treatment, day and their interaction. In the case of a significant interaction term, differences in least squares means between day 1 and day 5 for each of the treatments and between treatments for each day were calculated using an LSMESTIMATE statement.

The simulate adjustment for multiple comparisons was used in the case of the significant terms. The distribution of the conditional residuals for each model was evaluated to ensure the assumptions of normality and homoscedasticity for the statistical method had been met. Statistical analysis was not performed on the negative control group as repeat analysis was not performed in the negative control group. An alpha level of 0.05 was used to determine statistical significance.

Results

Analysis of Cyclosporine Effects on Feline Lymphocytes In Vitro

Both phorbol myristate acetate (PMA) and ionomycin were initially explored as agonists but failed to result in lymphocyte proliferation (data not shown). Five µg/mL ConA was therefore used for cell stimulation, as previously documented by Kuga et al., 2008. In the present study, there was a significant interaction between day and treatment ($P < 0.001$). ConA incubation with PBMCs resulted in a statistically significant increase in live cell counts at days 2, 3, 4, 5 and 6 compared to day 1 ($P < 0.001$). Maximal stimulatory effects of ConA occurred on day 5, as day 5 had significantly greater live cell counts than day 6 ($P = 0.0151$) (**Error! Reference source not found.**). Addition of cyclosporine to ConA-activated feline PBMCs resulted in a statistically significant decrease in live cell counts relative to when cells were exposed to ConA alone (**Error! Reference source not found.**). The decrease was significant at days 3, 4, 5 and 6 ($P < 0.001$), but a significant difference was not detected on day 1 ($P = 0.999$)

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or day 2 (P = 0.960). Minimal variability in individual cat responses to CsA was observed, as indicated by small standard error from the mean data.

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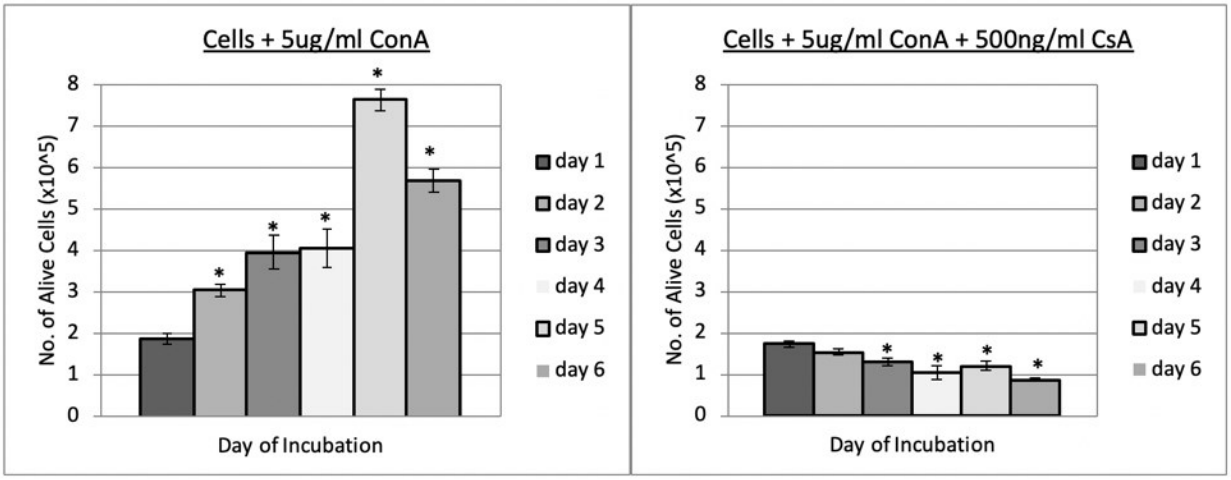

Figure 2.1 Cyclosporine inhibits lymphocyte proliferation in response to ConA

Exposure to 5µg/mL ConA results in proliferation of feline lymphocytes, with a peak of maximum proliferation on day 5 (A). Addition of 500ng/mL CsA results in a statistically significant suppression of lymphocyte proliferation on days 3, 4, 5 and 6 (B). Statistical analysis was performed on log10 transformed cell counts. Statistically significant differences in lymphocyte count relative to day 1 are indicated by *. The error bars indicate the standard error from the mean in lymphocyte counts amongst the 6 cats evaluated.

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Assessment of Cyclosporine Effects on Early and Late Apoptotic Changes in Feline PBMCs

Assessment of apoptosis in feline PBMCs via flow cytometry documented both early and late apoptotic changes in the cells exposed to cyclosporine (**Error! Reference source not found.**). There was a significant interaction between day and treatment for the percentage of live cells outcome ($P < 0.001$). Significant decreases in the percentage of live cells were evident at day 5 in PBMCs exposed to CsA alone ($P < 0.001$), ConA alone ($P = 0.002$), and ConA-activated PBMCs in the presence of CsA ($P < 0.001$) (**Error! Reference source not found.**). There was a significant interaction between day and treatment for the early apoptosis outcome ($P < 0.001$). There was a significant decrease in the percentage of early apoptotic cells in the PBMCs exposed to ConA ($P < 0.001$) (**Error! Reference source not found.**). Significant increases were evident in the percentage of early apoptotic cells exposed to CsA ($P < 0.001$). No significant change was identified with ConA-activated PBMCs in the presence of CsA ($P = 0.135$) for early apoptotic cells (**Error! Reference source not found.**). There was a significant interaction between day and treatment for the log10 late apoptosis outcome ($P < 0.001$). Significant increases in the percentage of log10 late apoptotic cells were shown in all experimental groups ($P < 0.001$), with the most dramatic increases in PBMCs treated with both ConA and CsA (**Error! Reference source not found.**). Finally, there was a significant interaction between day and treatment for the log10 necrosis outcome ($P = 0.027$). Significant increases in the percentage of necrotic cells were documented at day 5 in PBMCs exposed to ConA, CsA and both reagents ($P < 0.001$) (**Error! Reference source not found.**).

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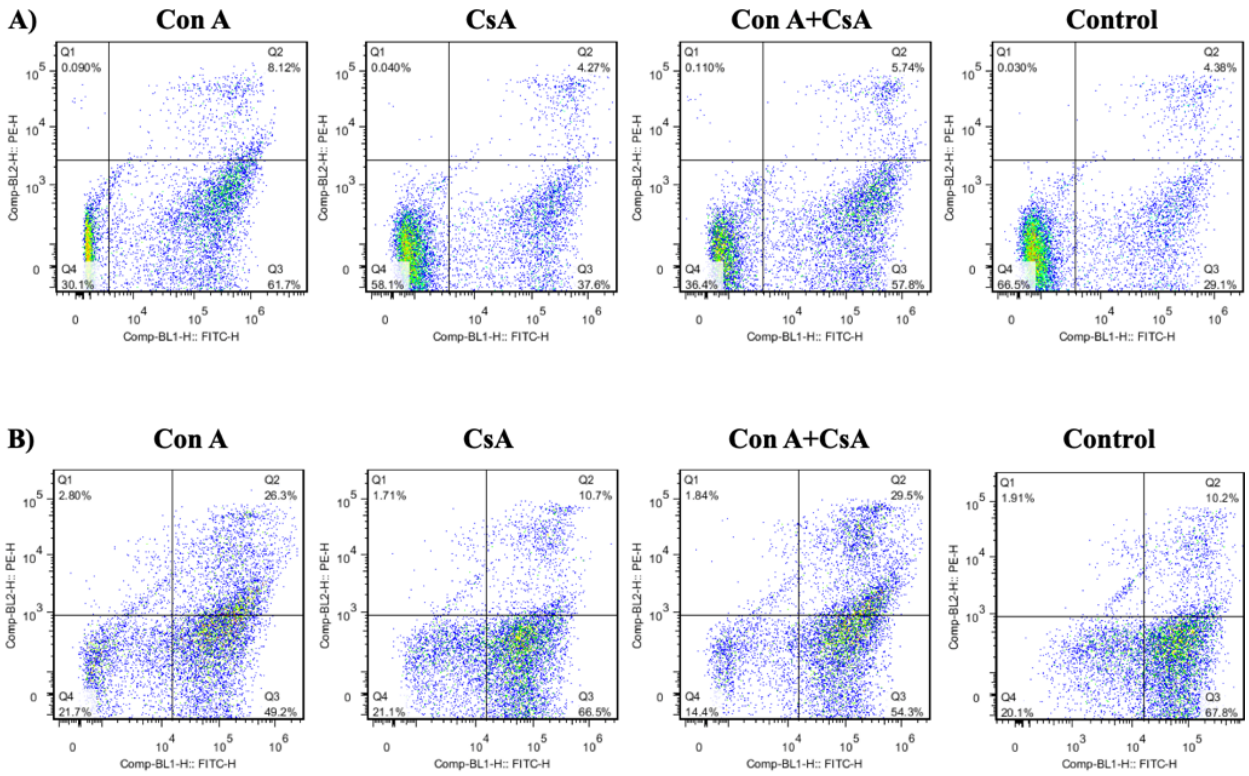

Figure 2.2 Apoptotic changes in feline PBMCs exposed to cyclosporine by flow cytometry

Apoptosis assay results on day 1 (A) and day 5 (B) in response to incubation with 5µg/mL ConA, 500ng/mL CsA, and 5µg/mL ConA + 500ng/mL CsA respectively. Results are displayed as a percentage of total cell count. Cells without an activator or inhibitor was used as a negative control. Q1 – necrosis, Q2 – late apoptosis, Q3 – early apoptosis, Q4 – live cells.

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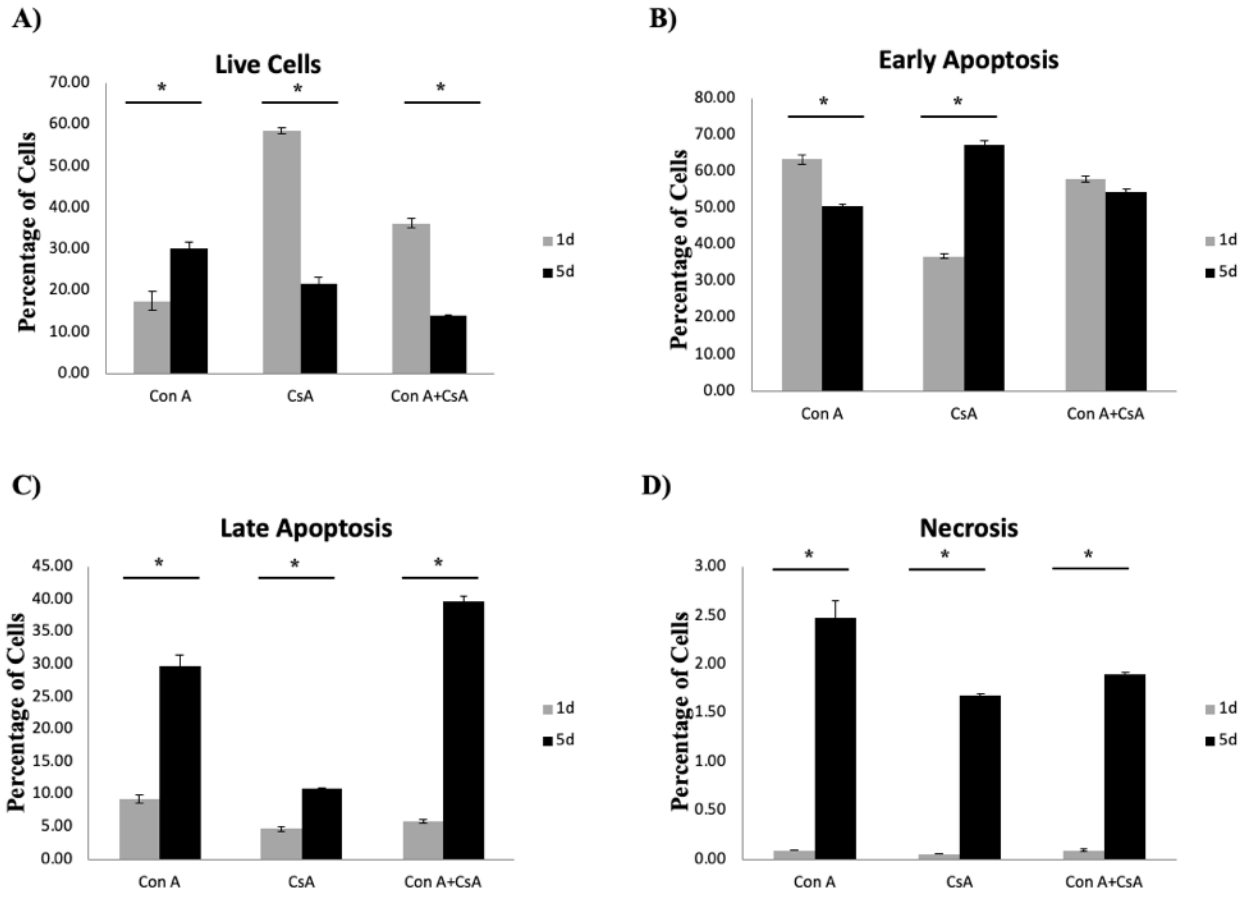

Figure 2.3 Figure A.1 Statistical interpretation of early and late apoptotic changes in feline PBMCs

Statistical interpretation of apoptosis assay in response to incubation with 5µg/mL ConA, 500ng/mL CsA, and 5µg/mL ConA + 500ng/mL CsA respectively. Statistical analysis was not performed on the negative control group as repeat analysis was not performed in the negative control group. Results are displayed as a percentage of total cell count. Three biological replicates were performed. Statistically significant differences between day 1 and day 5 are indicated by *.

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Discussion

The results of our present study demonstrated that 5 µg/mL ConA effectively induced lymphocyte proliferation, with the maximal proliferation occurring on day 5 of incubation (5% CO_2 and 37°C). A ConA concentration of 5 µg/mL was selected based on previous literature[1]. Our activation protocol differs from a previous study that utilized a similar concentration of ConA (5 µg/mL) under different conditions (24 hours of incubation in 37°C in humidified air with 5% CO_2)[1]. Unlike our study, the previous study did not assess a temporal relationship between duration of exposure to activators and cell counts. Although PMA and ionomycin were initially studied as activators, based on reported effectiveness in previous canine studies[16,24,25], they failed to provide sufficient activation of lymphocytes. PMA, ionomycin and ConA were originally selected for evaluation as they are T-cell agonists. PMA activates the signal transduction enzyme protein kinase C, whilst ionomycin increases the intracellular level ofcalcium, thus stimulating T cells[26,27]. Concanavalin A, in contrast, binds and cross-links components of the T-cell receptor[28]. The differences in mechanisms of action of PMA, ionomycin and ConA may help explain the differences in species response to these T-cell activators. Interestingly, the previous study that documented T-cell activation in cats also used ConA[1]. Additional studies are required to determine if feline T lymphocytes are resistant to activation by PMA/ionomycin, or if further dose optimization of these two activators is required. The activation protocol developed in our study may provide a basis for future studies pharmacodynamically investigating the effects of immunosuppressive therapy on ConA-activated lymphocytes in cats.

Addition of cyclosporine to ConA-stimulated cells resulted in decreased live cell counts relative to those cells exposed to ConA alone. The dose of CsA was selected based on previous

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unpublished work by our research group. Previous studies have documented that CsA has no apparent cytotoxicity on feline lymphocytes, at drug concentrations from 10-1000 ng/mL, in the presence of ConA at 5µg/mL[1]. Our study confirms that, as in other species, CsA inhibits lymphocyte stimulation, as documented by live cell counts, in cats.

Immunosuppressive agents, including glucocorticoids and cyclosporine, have been shown to induce apoptosis in human cell lines[17]. Apoptosis is defined as biologically programmed cell death, characterized by nuclear DNA degradation, nuclear degeneration and condensation. Necrosis, in contrast, is defined as death of tissue caused by chemical or physical injury[29]. Our study confirms that CsA also induces both early and late apoptotic change in feline PBMCs as documented by flow cytometry. Furthermore, our data showed significant pro-apoptotic effects of CsA on resting and activated feline PBMCs. The apoptosis assay revealed a statistically significant increase in the percentage of cells undergoing early apoptosis when exposed to CsA. In addition, CsA induced late apoptosis. Cells exposed to both ConA and CsA had more dramatic late apoptotic effects than the group exposed to CsA alone. One potential explanation for this phenomenon is that cyclosporine acts in the G_0 phase of the cell cycle[30]. The increase in cell proliferation associated with ConA may increase the number of times a cell is in G_o and thus susceptible to cyclosporine. Horigome et. al. previously suggested that immunosuppression may be influenced by lymphocyte susceptibility to apoptosis, thus making apoptosis an interesting area of research when investigating cyclosporine-induced immunosuppression. Differences in susceptibility to apoptosis may play a key role in individual feline responses to cyclosporine. Further investigation is needed to determine the significance of individual variation in apoptosis induced by cyclosporine in cats, and whether this variability influences clinical response to immunosuppressive therapy.

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Pharmacodynamic monitoring of cyclosporine has been recommended in dogs due to marked differences in dog-to-dog responses to oral cyclosporine[3]. Cyclosporine is a calcineurin inhibitor that inhibits production of NFAT-regulated cytokines, including IL-2[15,16,24]. IL-2 is essential for T-cell proliferation, and as such T-lymphocyte proliferation can be used as a surrogate marker of drug action. In fact, cell proliferation has been used as a marker of cyclosporine action in a number of other studies[16,24,25]. Our study demonstrated that cell proliferation appears to be viable as an indicator of cyclosporine action in cats. Although our study determined a viable protocol for ConA-induced proliferation of lymphocytes that was sensitive to the effects of cyclosporine, future studies would be needed to optimize the current activation protocol for use on whole blood or for use with different research technologies such as flow cytometric or RT-qPCR measurement of cytokine expression, to allow for the subsequent development of a more commercially feasible pharmacodynamic monitoring tool for cyclosporine in cats.

In conclusion, our study designed and optimized an activation protocol for feline lymphocytes with concanavalin A (5 µg/mL in 5% CO_2 at 37°C for 5 days) and documented that exposure to cyclosporine resulted in inhibition of cell proliferation, as noted by live cell counts. In addition, our study documented that cyclosporine induced apoptosis of lymphocytes in cats.

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CHAPTER III

ADDITIONAL EXPERIMENTS

1. Investigation into the use of Phorbol Myristate Acetate and Ionomycin as T-cell mitogens in Cats

Introduction

Pharmacodynamic assays evaluate the effect of a drug on a target cell or tissue and, in the case of cyclosporine, the target cell is the T lymphocyte. Many pharmacodynamic biomarkers of cyclosporine action have been investigated in humans and dogs, including lymphocyte proliferation, surface antigen expression, calcineurin activity and cytokine production[1,2,11,3–10]. Pharmacodynamic studies investigating cyclosporine in cats are limited. In 1987, Gregory et al. documented a non-cytotoxic decrease in lymphocyte response when cyclosporine was added to a whole-blood lymphocyte stimulation assay, thus indicating that cyclosporine has anti-lymphocytic properties in cats, as in other species[12]. Further work published in 2000 documented cyclosporine's anti-proliferative effects on feline lymphocytes via the use of various T-cell and mixed T-cell and B-cell mitogens, including concanavalin A (ConA) and pokeweed (PMW)[13]. Mitogens are required to induce maximal lymphocyte proliferation prior to assessing the effect of cyclosporine on T cells. However, little further research has been published on T-cell mitogens in cats. Preliminary studies performed by Kyles et al. indicated that Con A and PMW produced the greatest and most consistent degree of T cell proliferation in cats; however, to the author's knowledge data on other mitogens has not been published. In the Kyles et al. study, 46*nM* of cyclosporine was sufficient to cause a 50% reduction in ConA-induced T cell

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proliferation, whereas 33*nM* of cyclosporine was sufficient to cause a 50% reduction in PMW-induced proliferation, thus suggesting that Con A may be the most suitable activator in cats. Since PMW stimulates both B and T cells, the exact degree of proliferation of B versus T lymphocytes was undetermined[14]. In dogs, phorbol myristate acetate (PMA) and ionomycin are used as T-cell mitogens in pharmacodynamic monitoring of cyclosporine; however, they have not been investigated in cats. A protocol for maximal mitogen-induced T cell activation in cats is required to allow for subsequent pharmacodynamic monitoring of cyclosporine therapy. Thus, the aims of this experiment were to determine if PMA and ionomycin were suitable for activation of T lymphocytes in cats and, if successful, to determine appropriate concentrations of PMA and ionomycin for maximal T-lymphocyte NFAT-regulated cytokine expression and lymphocyte proliferation.

Materials and Methods

Animals

Twelve client-owned (9 male neutered and 3 female spayed) cats with a mean (± SD) body weight of 5.18 ± 1.0 kg were used for this study. The following breeds were represented: domestic shorthair (n=9), domestic longhair (n=2) and Siamese (n=1). Cats were determined to be healthy based on pre-enrollment physical examination in addition to CBC, serum chemistry and infectious disease testing. The experimental protocol was approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC).

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T-cell Mitogens

Stock solutions of PMA (10 mg/mL) and ionomycin (1mM) from Sigma-Aldrich (St. Louis, MO) were made in 100% ethanol and were stored at -70°C. Samples were slowly warmed to 20°C prior to use and were diluted 1:100 in sterile saline for PMA and 1:1 for ionomycin.

Experiment 1 – Whole Blood Collection

For blood collection, each cat was placed on a table and gently restrained in either right or left lateral recumbency or held sitting with the neck extended. The left or right jugular vein was clipped and prepped with alcohol. The jugular vein was manually occluded, and a 22-gauge needle attached to a 10cc syringe was inserted into the jugular vein. 5ml of blood was collected into heparinized tubes

Experiment 1 – T cell Activation

Half of the sample were activated with PMA and ionomycin at the following concentrations; PMA 10 ng/mL, 12.5 ng/mL, 15 ng/mL and ionomycin 0.6 µM, 0.8 µM, 1.0 µM (see Tables 2 and 3), whilst the remaining samples were untreated. Each concentration was performed in triplicate on each patient. These concentrations were selected based on previous studies in dogs[11]. The mixed samples were then incubated at 37°C with 5% CO_2 for 5 hours, based on a previous study[9]

Experiment 1 – RNA Extraction from Whole Blood

RNA extraction was performed using a commercial RNA extraction kit[2] as detailed below;

1. One ml of whole blood was mixed with 5ml of erythrocyte lysis buffer in a 15ml Falcon tube

[2] Qiagen RNA Blood Mini Kit, Valencia, CA 91355

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2. The sample was then incubated on ice for 15 minutes, and vortexed briefly at 5 and 10 minutes
3. The sample was then centrifuged at 400xg for 16 minutes at 4°C, and the supernatant was discarded
4. Two ml of erythrocyte lysis buffer was then added to the cell pellet and the pellet was resuspended by vortexing
5. The sample was then centrifuged at 400xg for 16 minutes at 4°C, and the supernatant was discarded.
6. Six-hundred µL of Buffer RLT® was then added to the pellet
7. The sample was then pipetted onto a QIAshredder® spin column placed inside a 2ml collection tube
8. The tubes were then centrifuged for 2 minutes at 400xg. The spin column was then discarded, and the run-through was collected.
9. Six-hundred µL of 70% ethanol was added to the sample. The sample was then placed on a new QIAamp® spin column.
10. The spin column was then centrifuged at 8000xg for 1 minute and the flow-though was discarded.
11. Six-hundred µL of sample was then placed onto the same QIAamp® spin column
12. The sample was centrifuged at 8000xg for 1 minute and the flow through was discarded
13. Three hundred and fifty µL of Buffer RW1® was then added to the QIAamp® spin column
14. The sample was then centrifuged at 8000xg for 1 minute and the flow through was discarded
15. Ten µL of DNase stock solution was then added to 70µL of Buffer RDD® immediately prior to use

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16. The DNase mixture (80µL) was then added to a QIAamp® spin column, and sample was incubated at room temperature for 15 minutes
17. Three-hunded and fifty µL of buffer RW1® was then added to the QIAamp® spin column
18. The sample was then centrifuged at 8000xg for 1 minute and the flow through was discarded
19. A QIAamp® spin column was then added to a new 2ml collection tube. Five-hundred µL of buffer RPE® was then added to the QIAamp® spin column
20. The sample was centrifuged at 8000xg for 1 minute and once again the flow through was discarded
21. Five hundred µL of buffer RPE® was added to a new QIAamp® spin column, and the sample was centrifuged at 8000xg for 3 minutes and the flow through was discarded.
22. The column was placed in a new 2ml collection tube and was centrifuged at 8000xg for 1 minute
23. The QIAamp® spin column was then transferred to a 1.5ml microcentrifuge tube. Fifty µL of RNAase free water was then added to the column and centrifuged at 8000xg for 1 minute
24. The samples were stored on ice until RNA quantification

Experiment 2 – Peripheral Blood Mononuclear Cell Isolation

PBMCs were immediately isolated from whole blood by density gradient centrifugation using Histopaque®-1077, as previously described in dogs[15]. Following isolation, PBMCs were reconstituted with an equal volume of complete medium (CM). CM consisted of 9mL of RPMI-1640[3] (Sigma Aldrich, St. Louis, MO) and 1mL of heat-inactivated fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA). 0.1mL of an antibiotic antimycotic solution (Sigma Aldrich,

[3] Sigma Aldrich, St. Louis, MO

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St. Louis, MO) was also added to the culture medium (1000U penicillin, 1mg streptomycin and 2.5mg amphotericin B). PBMC's were initially utilized to prevent interference from other cell populations

Experiment 2 – RNA Extraction from PBMC's

RNA extraction was performed as per the whole blood protocol above, with the exception of erythrocyte lysis (Buffer EL) steps, which were excluded.

RNA Quantification

RNA quantification was performed using a Thermo Scientific Nano Drop One (Unity Lab Services, Madison, WI), according to manufacturer instructions. Briefly, 1µl of RNAase free water (Qiagen, Germantown, MD) was used to calibrate the machine prior to use. 1µl of each sample was then applied to the reading arm and each sample was recorded in triplicate. If a greater than 10% difference between results was noted, a fourth reading was performed, and the discordant data was excluded. A260:A280 ratio of 1.8 – 2.0 indicated no contamination; however, samples outside of this range were suspected to be contaminated and repeat analysis was performed on such samples.

PCR Analysis

Feline CsA qPCR Primers

The following NFAT-regulated cytokines were selected for PCR analysis; IL-2, IL-4, IFN-gamma. GAPDH was used as a housekeeping gene. The primer sequences are listed in Table 3.1 below and were selected based on a prior study[16].

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Table 3.1 Primer sequences for PCR analysis

Primer	Primer Sequence
IL-2 Forward	CGG TTG CTT TTG AAT GGA GTT AA
IL-2 Reverse	TTA AAT GTG AGC ATC CTG GAG AGT T
IL-4 Forward	CGT CTT GGC AGC CCC TAA G
IL-4 Reverse	CGG TTG TGG CTC TGC AGA A
IFN-gamma Forward	AGG AGC ATG GAC ACC ATC AAG
IFN-gamma Reverse	CCC GTT TAC TGG AGC TGG TAT T
GAPDH Forward	AAA TTC CAC GGC ACA GTC AAG
GAPDH Reverse	TGA TGG GCT TTC CAT TGA TGA

Primer sequences for various NFAT-regulated cytokines. GAPDH was used as a housekeeping gene.

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PCR Settings

Thermal profile settings were adapted from canine studies and from Invitrogen SuperScript® III Platinum SYBR Green One Step qRT-PCR manufacturer instructions[9]. The thermal profile for the PCR analysis was as follows; 50°C for 3 minutes, 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds, followed by 40°C for 1 minute. Melting curve analysis was performed as followed; 40°C for 1 minute, 95°C for 1 minute, 55°C for 30 seconds and 95°C for 30 seconds.

Data Analysis

The relative change in gene expression of the samples was calculated using the delta CT method with GAPDH as a reference gene as previously described[9]. A brief summary is provided below:

1. The 'reference' score for each gene was calculated as below;
    - IL-2 Reference Score = Control UNA IL2 – Control ACT IL2
    - IL-4 Reference Score = Control UNA IL2 – Control ACT 1L2
    - IFN-gamma Reference Score = Control UNA IL2 – Control ACT IL2
2. The delta CT value was then calculated as follows;\
    - delta CT for IL-2 = GAPDH CT – IL2 CT
    - delta CT for IL-4 = GAPDH CT – IL4 CT
    - delta CT for IFN-gamma = GAPDH CT – IFN-gamma CT
3. For each patient, the difference between activated (ACT) and unactivated (UNA) CT values were then calculated for each reference gene as shown below;
    - IL-2 calculated difference = Patient UNA IL2 – Patient ACT IL2
    - IL-4 calculated difference = Patient UNA IL4 – Patient ACT IL4

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- IFN-gamma calculated difference = Patient UNA IFN-gamma – Patient ACT IFN-gamma

Results

PMA and ionomycin, at the concentrations documented previously to activate T cells in dogs, failed to increase total nucleic acid concentration in feline lymphocytes [8]. In addition, dose adjustments in PMA and ionomycin failed to achieve increases in total nucleic acid concentration. See Table 3.2 and Table 3.3. PCR analysis failed to document a significant increase in gene expression of the NFAT-regulated cytokines IL-2, IL-4, and IFN-gamma as determined by delta CT analysis. See Table 3.4

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Table 3.2 Nucleic acid concentrations in unactivated and activated feline samples with the mitogens PMA and ionomycin at varied concentrations (whole blood).

PMA Concentration (ng/mL)	Ionomycin Concentration (µM)	Mean ± SD Nucleic Acid Conc (ng/µL)
0.0	0.0	2.14 ± 0.32
10.0	0.6	1.30 ± 0.06
10.0	0.8	2.05 ± 0.13
12.5	0.6	1.23 ± 0.17
12.5	0.8	1.62 ± 0.21
12.5	1.0	1.84 ± 0.29
15.0	0.8	1.45 ± 0.04
15.0	1.0	2.16 ± 0.08

The mean concentration of nucleic acid and the standard deviation around the mean are recorded following RNA extraction from activated feline lymphocytes. The sample used was whole blood. Incubation with PMA and ionomycin failed to result in consistent detectable increases in nucleic acid concentration

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Table 3.3 Nucleic acid concentrations in unactivated and activated feline samples with the mitogens PMA and ionomycin at varied concentrations (PBMCs).

PMA Concentration (ng/mL)	Ionomycin Concentration (μM)	Mean ± SD Nucleic Acid Conc (ng/μL)
0.0	0.0	2.21 ± 0.26
10.0	0.6	1.71 ± 0.34
10.0	0.8	2.02 ± 0.07
12.5	0.6	2.43 ± 0.11
12.5	0.8	1.92 ± 0.10
12.5	1.0	1.82 ± 0.17
15.0	0.8	1.41 ± 0.10
15.0	1.0	2.07 ± 0.14

The mean concentration of nucleic acid and the standard deviation around the mean are recorded following RNA extraction from activated feline lymphocytes. The sample used was feline PBMCs. Incubation with PMA and ionomycin failed to result in consistent detectable increases in nucleic acid concentration.

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Table 3.4 Change in cytokine expression of NFAT-regulated cytokines following activation with PMA and ionomycin at varied concentrations.

Cytokine	PMA Concentration (ng/mL)	Ionomycin Concentration (µM)	Delta CT
IL-2	10.0	0.6	0.32
IL-2	12.5	0.6	2.62
IL-2	15.0	0.6	0.40
IL-2	10.0	0.8	-2.60
IL-2	12.5	0.8	0.48
IL-2	15.0	0.8	0.61
IL-2	10.0	1.0	0.34
IL-2	12.5	1.0	-3.20
IL-2	15.0	1.0	-4.26
IL-4	10.0	0.6	0.22
IL-4	12.5	0.6	-4.70
IL-4	15.0	0.6	0.32
IL-4	10.0	0.8	-2.60
IL-4	12.5	0.8	0.48
IL-4	15.0	0.8	-2.11
IL-4	12.5	1.0	-0.01
IL-4	15.0	1.0	-4.26
IFN-γ	10.0	0.6	-0.12
IFN-γ	10.0	0.8	-3.86
IFN-γ	12.5	0.6	-5.14

Incubation with PMA and ionomycin failed to result in significant detectable increases in expression of various NFAT regulated cytokines

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Discussion

In this study, we documented that PMA and ionomycin at the following concentrations; PMA 10.0-15.0 ng/mL and ionomycin 0.0-1.0µM, appeared to fail to sufficiently activate feline lymphocytes in whole blood or isolated PBMCs, as determined by RNA quantification and PCR analysis in unactivated and activated samples. In dogs, PMA at 12.5 ng/mL and ionomycin at 0.8 µM are frequently used for T-lymphocyte stimulation[8,9,17]

Potential explanations for the failure of PMA and ionomycin to detectably activate feline T lymphocytes in this study include species differences in response to mitogens, the use of insufficient doses of mitogen and the use of inappropriate incubation conditions. PMA, ionomycin and ConA have different mechanisms of action, and this may help to explain species differences in response to each mitogen. PMA activates protein kinase C, whereas ionomycin increases intracellular calcium concentration, both of which ultimately result in T-lymphocyte stimulation[18,19]. Interestingly, previous studies in cats have uniformly utilized the mitogens ConA or pokeweed, and unpublished preliminary studies by Kyles et al. suggest that ConA and pokeweed are the most successful T lymphocyte activators in cats[12,13]. ConA has a direct effect on the T-cell receptor leading to down-stream signal transduction[20]. Pokeweed, in contrast to ConA, has been shown to stimulate both B and T lymphocytes in humans[14]. Further potential explanations include failure to detect the amplified RNA sequence. This can relate to failure of the primer sequences to anneal to the target RNA strand. The primers selected; however, had previously been used successfully in a feline qRT-PCR study[16], thus promoting consideration of technological issues. Potential technical issues include an inadequate annealing temperature or annealing time. Additional potential considerations include the presence of introns between

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primer sites resulting in incomplete product or failure to amplify, and hair-pinning of primers due to compatible sequences with each primer.

Future studies should evaluate the primer sequences utilized in this study to determine whether the mitogens utilized failed to activate T lymphocytes, or whether the study failed due to inability to detect amplified RNA sequences. Future studies should also investigate a dose titration of other mitogens, such as ConA or pokeweed, for maximal stimulation of feline lymphocytes. The ultimate long-term goal would be to generate a commercially available tool for pharmacodynamic monitoring of cyclosporine in cats.

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2. Concanavalin A Titration Using Flow Cytometry

Introduction

Con A is a T-lymphocyte mitogen commonly utilized in cats, and its mechanism of action is via direct stimulation of the T-lymphocyte receptor[12,13,20]. However, dose optimization is required to maximize T-lymphocyte stimulation *in vitro*. In this brief study, we utilized flow cytometry to determine the most suitable concentration of Con A for stimulation of feline PBMCs.

Materials and Methods

Animals

Two client-owned (both female spayed) cats with a mean (± SD) body weight of 4.4 ± 0.71 kg were used for this study. Both cats were of the domestic shorthair breed. Cats were determined to be healthy based on pre-enrollment physical examination in addition to CBC, serum chemistry and infectious disease testing. The experimental protocol was approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC).

Incubation and Activation Protocols

Serial dilutions of Con A (Sigma Aldrich, St. Louis, MO) were produced via dilution with CM. CM was formulated as documented in the previous experiment. The following concentrations of ConA were produced: 20µg/mL, 10µg/mL, 5µg/mL, 2.5µg/mL, and 1.25µg/mL. A negative control consisting of CM was also utilized. The concentrations were selected due to prior successful use of 5µg/mL[16].

PBMCs were isolated as documented in the previous experiment. PBMC's were then incubated with the following concentrations of ConA: 0µg/mL (control), 1.25µg/mL, 2.5µg/mL,

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5µg/mL, 10µg/mL and 20µg/mL. The incubation conditions were as follows: 37°C and 5% CO_2 and 10 hours of incubation.

Flow Cytometry

After incubation, 2mL of suspension from each well was transferred into flow cytometric tubes (Sigma Aldrich, St. Louis, MO). The sample was then centrifuged at 500xg for 5 minutes. The supernatant was decanted into a waste receptacle and the sample was washed with 2mL of PBS. This process was repeated twice. 3µL of LIVE/DEAD fixable dead cell stain was then added to the sample, and the sample was vortexed lightly to mix the contents. The sample was then incubated for 30 minutes at room temperature. The sample was then centrifuged at 500xg for 5 minutes, and the supernatant was decanted. The sample was then washed with PBS as above. Finally, the sample was re-suspended in complete media and passed through a 35-micron filter to remove any clot material. The material was then analyzed by flow cytometry using the following lasers/filters;

- 405nm laser with the 530/30 bandpass filter (LIVE/DEAD aqua)
- Threshold = 300,000 cells
- Collect = 25,000 cells
- V Channel 2
- Slow setting

Results

The samples from both cats incubated with 20µg/mL ConA visibly clotted, likely indicating cell death. These samples were not submitted for flow cytometry analysis to prevent inadvertent damage to the machine. Analysis of flow cytometry data revealed that 5µg/mL and

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10µg/mL ConA had similar activation, but 10µg/mL ConA had lower viability. Cat two had an abnormal scatter pattern and was excluded from analysis.

Discussion

Flow cytometry was utilized to assess PBMC activation in response to ConA stimulation. Our study found that 5µg/mL of ConA was the most suitable choice for PBMC activation in cats. This dose is the same as in a previous study[16].

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CHAPTER IV

CONCLUSIONS AND FUTURE WORK

This thesis details the initial investigations and groundwork required for further studies into pharmacodynamic monitoring of cyclosporine therapy in cats. Our research initially evaluated PMA and ionomycin as T-cell mitogens, given their success in canine pharmacodynamic assays. However, PMA and ionomycin at various concentrations failed to provide sufficient activation of T lymphocytes and were therefore not suitable for subsequent analysis with cytokine primers. We subsequently turned our attentions to ConA as a potential T cell mitogen. Our initial experiment evaluated titrations of ConA, with evaluated concentrations centered around a previously published concentration, and the data supported the use of 5µg/mL of ConA for T-lymphocyte activation in cats. This concentration of ConA was subsequently used to stimulate PBMCs as part of a cell proliferation study, in which feline PBMCs were quantified daily for 6 days, via trypan blue exclusion dye. Alive and necrotic cell counts were performed on cell cultures exposed to Con A and cyclosporine. This study documented an optimal activation time of 5 days for cyclosporine in cats, much longer than previously published[1]. This study also confirmed the findings of a previous study, and documented cyclosporine's anti-lymphocytic properties in cats. The results of the above *in vitro* studies when considered in unison suggest a suitable activation protocol for T lymphocytes in cats would comprise of 5% $C0_2$ with a 5-day incubation time with 5µg/mL ConA. This protocol may provide a basis for future pharmacodynamic studies evaluating lymphocytes in cats. Future studies should utilize the above

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activation protocol to evaluate expression of NFAT-regulated cytokines, and their response to cyclosporine therapy.

Preliminary studies as part of this project investigated measurement of IL-2, IL-4, IFN-gamma and the housekeeping enzyme GAPDH (nucleotide sequences listed in Table 4) using a quantitative reverse transcription PCR as documented by Kuga et al[1]. However, in our study, detection of cytokines was unsuccessful. Potential explanations for this include the presence of introns between primer sites resulting in incomplete product and failure to amplify, hair-pinning of primers due to compatible sequences within each primer or primer-dimerization, and potentially incompatible PCR parameters[2]. However ,the PCR parameters utilized in our study were based on the previously successful work by Kuga et al[1].

Given the failure of pharmacokinetic monitoring tools to correlate with clinical immunosuppression, and the lack of consensus regarding therapeutic cyclosporine blood levels, pharmacodynamic monitoring offers much promise in feline medicine[3,4]. Following the development of an optimal T lymphocyte activation protocol as part of this research, and prior documentation of successful measurement of NFAT-regulated cytokines via PCR in cats[1], a PCR-based technique probably offers the most promise in the development of a commercial pharmacodynamic monitoring tool for cyclosporine in cats. However, further optimization and validation of a quantitative reverse transcription PCR for measurement of various NFAT-regulated cytokines in cats is required prior to commercial utilization. Transition to a whole blood-based assay is also likely indicated prior to commercial use

Pharmacodynamic monitoring techniques would not only assist in the management of individual patients, but could also be used to investigate suitable starting doses of cyclosporine for systemic immunosuppression in cats. The current lack of consensus on the ideal dose of

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cyclosporine for systemic immunosuppression in cats is clear when evaluating prior studies. Starting dose ranges of 5-20mg/kg/day have been documented, with a dose frequency of either every 12 hours or every 24 hours[5–13]. In dogs suffering from acute life-threatening immune-mediated disease, a dosage of 5-10mg/kg PO q 12h is recommended, whereas a dosage of 5mg/kg PO q 24h is recommended for most dermatological indications[14]. It is likely that twice daily administration of cyclosporine is also required in cats for systemic immunosuppression, given prior reports; however, additional research is required to confirm these anecdotal suspicions and small case reports.

Although not studied here, additional research areas for pharmacodynamic monitoring of cyclosporine in cats may include assessment of proliferation assays, surface antigens as markers of T cell activation and calcineurin inhibition assays, as previously studied in dogs and humans

Cyclosporine has been shown to induce apoptosis in human mitogen-activated PBMC's; however, this had not previously been studied in cats[15]. During this project, we investigated the effects of cyclosporine on lymphocyte apoptosis. To our knowledge, this is the first project to document lymphocyte apoptosis associated with cyclosporine therapy in cats. We utilized an Annexin-V apoptosis detection kit (BioVision Inc, Mountain View, CA) as previously described[16] and noted both early and late apoptotic change in feline PBMC's. Although definitive conclusions are limited by animal numbers, we also documented marked individual variation in the apoptotic response of PBMCs to cyclosporine. This may help explain variations in individual patient responses to cyclosporine. Future studies should evaluate apoptotic responses of PBMCs to cyclosporine in a larger group of cats. If marked variation is noted, *in vivo* assessment of apoptosis and its correlation with clinical immunosuppression should be considered; however, the use of flow cytometry, which is too cumbersome for routine clinical

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use, to assess apoptosis limits the commercial application of apoptotic monitoring of cyclosporine therapy in cats.

Although pharmacokinetic monitoring of cyclosporine is not recommended when treating skin disease[17], veterinarians should remain vigilant to marked unpredictable immunosuppression in individual patients. If patients appear to be overtly immunosuppressed, it is the author's opinion that measurement of peak or trough cyclosporine blood levels should be considered until further pharmacodynamic monitoring tools are developed. However, if the results of pharmacokinetic monitoring do not correlate with clinical suspicion, veterinarians should consider the previously documented limitations with such techniques in cats.

This thesis describes preliminary studies into pharmacodynamic monitoring of cyclosporine in cats and, with further investigation, pharmacodynamic monitoring may become a commonly utilized tool in the balance between maintaining sufficient immunosuppression and avoidance of life-threatening secondary infections such as toxoplasmosis in cats receiving immunosuppressive doses of cyclosporine.

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