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THE EFFECTS OF PYRANTEL TARTRATE (STRONGID®-C)
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THE EFFECTS OF PYRANTEL TARTRATE (STRONGID®-C) ON *SARCOCYSTIS NEURONA*

By

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ABSTRACT

THE EFFECTS OF PYRANTEL TARTRATE (STRONGID®-C) ON *SARCOCYSTIS NEURONA*

By

Elizabeth Ann Kruttlin, DVM

This thesis describes the effects of pyrantel tartrate (Strongid®-C) on *Sarcocystis neurona* merozoites and sporozoites both *in vitro* and in a gamma interferon knock-out mouse model of the disease equine protozoal myeloencephalitis (EPM). Anecdotal reports from private practicing veterinarinans suggest that horses fed daily Strongid®-C showed a lower incidence of clinical EPM than those not fed the dewormer, leading us to formulate the hypothesis that pyrantel tartrate affects the viability of *S. neurona.*

The results of this study show pyrantel tartrate, at concentrations greater than 2.5 mM, is 100% lethal to *S. neurona* merozoites. Pyrantel tartrate at concentrations greater than 5 mM is lethal to *S. neurona* sporozoites. Pyrantel tartrate given orally to gamma interferon knock-out mice at the dose of 2.5 mM increased the mean survival time of mice. Acetylcholine (ACh) chloride, at concentrations similar to that of pyrantel tartrate, did not produce the same lethal consequences in *S. neurona* merozoites, indicating that the drug is unlikely to work at cholinergic receptors in this parasite.

From these studies, we concluded that pyrantel tartrate has the potential to be effective against the merozoite and sporozoite stages of *S. neurona*, if high enough concentrations of the drug can be delivered effectively *in vivo*. Efficacy against the parasite *in vitro* was demonstrated to be dose dependent and repeatable in cell culture.

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LITERATURE REVIEW AND STUDY OBJECTIVES

1.1 Equine Protozoal Myeloencephalitis (EPM)

1.11 Introduction and History:

Equine protozoal myeloencephalitis (EPM) is a progressive neurologic disease of equids. The clinical syndrome was first described as "segmental myelitis" by Rooney et al in 1970[1], but the name "equine protozoal myeloencephalitis", coined by Mayhew et al. in 1976[2], gained favor after Cusick et al. reported the presence of protozoa in lesions from affected horses[3]. First believed to be caused by *Toxoplasma gondii*[3], conclusive evidence that EPM was caused by a *Sarcocystis* species was provided by Simpson and Mayhew in 1980[4].

1.12 Clinical signs:

EPM is described as a progressively debilitating disease affecting the central nervous system (CNS) of horses[5,6,7,8,9]. Clinical signs often range from acute to insidious onset of focal or multifocal signs of neurologic disease. The brain, brainstem, spinal cord, or any combination of these areas can be involved. Affected horses also may have abnormal upper airway function, seizures, or unusual or atypical lameness. Gait abnormalities are a result of damage to the spinal cord and can be variable depending on severity and location of lesions. Physical examination of affected animals generally shows vital signs to be normal; however, some horses may be thin and mildly depressed. Most horses also have normal complete blood count and serum chemistry values.

Many other neurologic diseases can manifest clinical signs similar to those of EPM[10]. Examples include cervical vertebral myelopathy (CVM), equine herpes virus myelitis (EHV-1), equine motor neuron disease (EMND), rabies, tetanus, neoplasia, leukoencephalomalacia (moldy corn poisoning), equine viral encephalomyelitis, trauma to the CNS, or multi-focal bacterial encephalitis. One of the most useful clinical signs in establishing the diagnosis of EPM is that horses with the disease often have asymmetric gait deficits with focal muscle atrophy[5,6,7,8,9]. In order for clinicians to make the proper diagnosis of EPM, all previously mentioned diseases should be considered and ruled out based on clinical signs and diagnostic testing.

1.13 Causative agent:

Sarcocystis neurona is the primary etiologic agent of EPM[4,11,12,13,14]. *Neospora hughesi* has also been identified in four cases of EPM[15,16,17,18], even though the majority of cases of EPM continue to result from *S. neurona* infection.

The lifecycle of *Sarcocystis* sp. is complex and involves two hosts. The sarcocyst stage of the parasite is found within muscle tissue of the intermediate host and is consumed by the definitive host. Upon digestion, the cyst wall is broken down and the bradyzoite stage of the parasite is released. Bradyzoites penetrate the host's intestinal epithelium and develop into the sexual stages of the parasite, microgametes and macrogametes. The microgamete fertilizes the macrogamete, resulting in a zygote which matures to become an oocyst containing two sporocysts. The oocyst is released from its host cell, travels down the intestine until the oocyst wall is ruptured, and sporocysts are excreted in the feces of the definitive host[19,20].

The intermediate host becomes infected by ingesting the sporocyst stage of the parasite from fecal matter or food or water contaminated with fecal matter. Upon ingestion, sporocysts release sporozoites within the gastrointestinal (GI) tract of the host. Sporozoites penetrate the gut and migrate to endothelial cells of the host's internal organs. In most cases, sporozoites undergo two rounds of asexual schizogony and become tachyzoites, then motile merozoites. Merozoites are released from schizonts and travel in circulating lymphocytes to muscle tissue. In muscle, they undergo fixed rounds of merogony (asexual replication) and develop into bradyzoites in intramuscular cysts. Sarcocysts contain large numbers of tightly packed bradyzoites that are infective to the definitive host. The lifecycle is continued when a definitive host ingests the muscle tissue as prey or carrion, and the bradyzoites emerge in the host's intestine[19,20].

Recently, the lifecycle of *S. neurona* has been completed. The North American opossum (*Didelphis virginiana*) is the definitive host of *S. neurona*[21,12]. Additionally, the South American opossum (*Didelphis albiventris*)also has been shown to act as a definitive host[22]. The parasite has been shown to utilize a variety of intermediate hosts including the nine-banded armadillo (*Dasypus novemcinctus*)[23,24], the striped skunk (*Mephitis mephitis*)[25], the raccoon (*Procyon lotor*)[26], and the domestic cat (*Felis domesticus*)[27]. Horses are considered aberrant hosts of *S. neurona*[11,28], and until recently, it was believed that the parasite could not undergo its final stage of cyst formation in this host. However, a recent study by Mullaney et al. found both mature schizonts in the brain and spinal cord and mature sarcocysts in the tongue of a young colt with neurologic disease consistent with EPM[29]. Sarcocysts were found to have features identical to published features of *S. neurona* on electron microscopy and produced *Sarcocystis*

specific PCR products. RFLP analysis of these products showed banding patterns characteristic for *S. neurona*. While this evidence is highly suggestive that horses have the potential to act as intermediate hosts, further studies are needed to demonstrate Koch's postulates before this claim can be made.

1.14 Pathogenesis:

The pathogenesis of EPM is not clear. It is assumed that horses are infected with *S. neurona* by consuming sporocysts in contaminated hay, grain, grass, or water[7]. Studies in gamma interferon knock-out mice orally inoculated with *S. neurona* sporocysts indicate that the parasite initially replicates to a limited extent in visceral tissues and then is transported to the CNS probably via leukocytes[30]. The development of a reliable equine model is necessary to study the pathogenesis of this disease. It is suggested that stress plays an important role in the pathogenesis of EPM, and inflicting a stressor, such as dexamethasone or transport, on horses prior to oral inoculation with *S. neurona* sporocysts has been shown to produce EPM in these animals[31]. However, the small number of test animals used this transport stress study does not provide solid evidence of a reliable equine model. Further studies are needed to demonstrate the infection dynamics of *S. neurona* in horses.

1.15 Pathology:

Sarcocystis neurona causes a non-suppurative inflammation of the CNS leading to gross lesions in both gray and white matter and necrosis of affected neural tissue[1,7,10,32,33]. Although the brainstem is more often involved than other brain areas,

lesions are more frequently seen in the spinal cord[1,10,32,33]. Perivascular cuffing by mononuclear cells is evident in some affected areas, particularly the meninges[1,10]. The inflammatory response is highly variable and can consist of infiltrates of a mixture of lymphocytes, neutrophils, eosinophils, macrophages, and multinucleate giant cells[34]. Degeneration of neurons and axons is commonly seen[1].

1.16 Diagnosis:

EPM remains the most commonly diagnosed infectious equine neurologic disease in America[34,35], and the Western blot test is the most commonly used diagnostic test for EPM in current use[9,36]. Western blot technique was developed in 1993; it detects antibodies in serum or cerebrospinal fluid[37]. In 2000, the test was enhanced by adding a blocking step in which blots are exposed to bovine anti-*Sarcocystis cruzi* IgG[38]. This treatment reduces false-positive results from antibody cross-reactivity to proteins common to *Sarcocystis* species, and has been found to have >99% sensitivity and 98% specificity[38]. A positive serological test indicates past or current infection with *S. neurona*, but does not confirm that the parasite has reached the CNS. For this reason, and given the high seroprevalence in clinically normal animals, testing of CSF is preferred[9,36]. A definitive diagnosis of EPM requires finding the parasite in neural tissue sections at postmortem examination or culturing the parasite from neural tissue[11,10,39]. Antemortem diagnosis of EPM should be made by combining the history and clinical picture of the animal in question with results of testing techniques described above.

A PCR test would help to decrease the number of false positive diagnoses of EPM because it can directly detect parasite DNA, rather than antibodies to the parasite that do not always suggest clinical disease. However, in clinical use, the test appears to suffer from low sensitivity because of the low number of merozoites present in the serum or CSF of the horse[40]. Parasite DNA may be easily missed. Techniques to collect and concentrate merozoites from large volumes of CSF (i.e. magnetic beads) would help to reduce the number of false negatives found with PCR and help to improve this test for clinical use.

1.17 Treatment:

Currently, one drug is FDA approved for the treatment of EPM in horses. Marquis™ (15% w/w ponazuril) given orally once daily at the dose of 5 mg/kg for 28 days has been found have activity against *S. neurona* in both laboratory and field settings[41,42]. Ponazuril is an anticoccidial triazine-based compound and is microbicidal to *S. neurona*. A similar drug, diclazuril, has been found to have anti-*S. neurona* activity in cell culture and may have some use as a prophylactic agent against *S. neurona* infections in horses[43,44]. Toltrazuril, an anti-coccidial drug used in several species, also has potential efficacy for the treatment of EPM[45].

Prior to approval of Marquis™, treatment was confined to the use of dihydrofolate reductase inhibitors such as sulfonamides and pyrimethamine[2,7]. Usual treatment involves the use of sulfadiazine at a dose of 20 mg/kg in combination with pyrimethamine at 1.0 mg/kg given orally once daily[2,7]. Treatment can be continued for 120 days or longer. A determination to discontinue treatment is based on either

significant improvement of clinical signs or the horse returning to normal and Western blot testing of CSF returning to negative[2,7]. This treatment regimen is not without risk. Prolonged antifolate therapy has been known to result in undesirable side effects including reduced spermatogenesis in stallions, harm to the fetus in pregnant mares, and bone marrow suppression in adult horses[9].

Nitazoxanide has broad spectrum activity against bacteria, protozoa, and helminthes and is effective against *S. neurona* in cell culture[46]. It has also recently been tested in a clinical field trial for the treatment of horses with EPM. The safety study indicated that, at a 2 X dose for 1 week, horses became lethargic, and at 4 X dosing, horses showed illness and one horse died[47]. The efficacy study of 70 horses showed 63% of horses as improving one grade or more or becoming negative on Western blot of CSF[47].

In addition to anti-protozoal drugs, anti-inflammatory drugs such as phenybutazone, dimethylsulfoxide and glucocorticosteroids may be used[7]. In situations where EPM is considered due to a poor immune response of the horse, use of immunostimulants such as levamisole, alpha-interferon, mycobacterial wall extract or killed *Propionibacterium acne* can also be considered[7].

1.18 Epidemiology:

EPM is the most commonly diagnosed equine protozoal disease in the eastern US[48]. It has not been shown to be contagious from horse to horse, and focal clusters of cases are unusual[33]. However, a study at The Ohio State University found that if EPM

had been diagnosed at a farm prior to diagnosis in one of the cases in the study, the risk of EPM was >2.5 times higher than if EPM had never been diagnosed previously[49].

EPM has been diagnosed in horses throughout North, Central, and South America[32]. Cases reported outside of this region were in horses imported from the Western hemisphere[7,32]. Prevalence of EPM in horses is estimated to be 0.5-1% of the horse population[53]; however, disease exposure is much higher with a 60% seroprevalence in some states[54]. The distribution of EPM follows the distribution of opossums, and the exact prevalence of *S. neurona* in opossums in unknown due to the absence of a simple method with which to identify *S. neurona* in opossum feces[50]. Based on completed surveys in Florida[51] and Michigan[52], the prevalence of *S. neurona* in opossums is considered to be very high.

EPM has been diagnosed in horses ranging in age from 2 months to 24 years[7,32]. More than 60% of cases in a retrospective study conducted in 1991 were reported in horses 4 years and younger[7]. There does not appear to be a breed or sex predilection[32]. Risk analysis has found that the risk for EPM was three times higher in spring and summer and six times higher in the fall when compared to winter[49]. This is believed to be due to climactic factors such as freezing temperatures affecting exposure and disease rates. Transport stress in relation to annual athletic competitions in the fall of the year may also contribute to the seasonal effect. A 2.5 X higher risk of EPM was present if opossums were observed on the property when compared to those barns where opossums were never seen[49]. A strong dose response relationship was seen between health events (i.e., aging, exercise, transport, injury, surgery, or parturition) and the risk for EPM[49].

These events may lead to immune suppression with subsequent development of clinical signs of EPM.

1.19 Rationale and justification for the study:

EPM is a devastating disease that is costly and often unrewarding to treat. Environmental exposure to *S. neurona* is difficult to prevent and occurs frequently based on seroprevalence. A logical means of controlling disease would be to focus on prevention. Currently, a killed, whole organism vaccine is available from Fort Dodge; however, it has limited approval, and safety and efficacy of the vaccine are questionable. Additionally, a vaccinated horse is indistinguishable from a non-vaccinated, exposed horse on serum Western blot, making disease diagnosis difficult.

Strongid®-C (pyrantel tartrate) is a daily anthelmintic feed additive used to prevent *Strongyle* infestation in horses. Pyrantel tartrate is an anthelmintic compound that possesses nicotine-like properties, acting similar to acetylcholine (ACh), and by inhibiting acetylcholinesterase[55]. These properties make the drug a depolarizing neuromuscular-blocking agent in susceptible parasites. Theoretically, daily pyrantel tartrate administration could prevent *S. neurona* infection in equids by killing sporozoites as they are excysted in the gut. If ACh receptors were found to be present in *S. neurona* sporozoites, it could be extrapolated that the drug works at a target similar to that of helminth worms; however, the role of ACh in protozoa yet to be defined.

Ancedotal reports from practitioners suggest horses fed Strongid®-C have a decreased incidence of clinical EPM. If the drug could stop *S. neurona* from passing into

the bloodstream of the horse, it could be used as a means of preventing *S. neurona* infection in equids.

1.2 Hypotheses:

It was hypothesized that:

- Strongyle larvae are able to attach to or ingest sporozoites and can serve as a transport host into the bloodstream of the horse.
- Pyrantel tartrate is lethal to *S. neurona* merozoites and sporozoites in vitro.
- Pyrantel tartrate prevents clinical disease in gamma interferon knock-out mice dosed orally with *S. neurona* sporocysts.
- *S. neurona* merozoites possess acetylcholine receptors similar to those of helminths, a possible drug target for pyrantel tartrate.

1.3 Study Objectives:

I. Determine if *Strongyle* larvae can attach to or ingest *S. neurona* sporozoites.

II. Develop an *in vitro* drug screening assay for *S. neurona* merozoites and sporozoites.

III. Determine the effects of pyrantel tartrate on *S. neurona* merozoites *in vitro*.

IV. Determine the effects of pyrantel tartrate on *S. neurona* sporozoites *in vitro*.

V. Determine the effects of pyrantel tartrate on *S. neurona* sporocyst infection in gamma interferon knock-out mice.

VI. Explore possible drug targets of pyrantel tartrate in *S. neurona* merozoites.

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CHAPTER 2

THE RELATIONSHIP BETWEEN *SARCOCYSTIS NEURONA* SPOROCYSTS AND STRONGYLE LARVAE

2.1 INTRODUCTION

The purpose of this series of experiments was to determine if *S. neurona* sporocysts can attach to or be ingested by first, second, or third stage *Strongyle* larvae. The pathogenesis of EPM is unclear. The route of migration of the parasite from the time of ingestion of sporocysts to the presence of merozoites in the CNS is unknown. It has been shown in gamma interferon knock-out mice fed *S. neurona* sporocysts that the parasite initially replicates to a limited extent in visceral tissues and is then transported to the CNS probably via leukocytes[1]. However, the possibility exists that sporocysts penetrate the GI tract and enter the bloodstream while attached to or while inside of *Strongyle* larvae and that pyrantel tartrate causes a decrease in the number of horses with EPM by killing the carrier of *S. neurona* (*Strongyle* larvae) and not by a direct effect on *Sarcocystis*.

2.2 MATERIALS AND METHODS

2.21 Strongyle collection and growth:

Fresh feces was collected from horses with natural *Strongyle* infections. The samples were collected directly after voidance from the animal, before hitting the ground, to prevent contamination with larvae from soil nematodes. A sucrose fecal floatation was performed to confirm the presence of healthy strongyle-type eggs. The fecal sample was then mixed in a 50:50 solution with autoclaved wood shavings in a metal bucket and distilled water added to the mixture until it became moist. Four subsamples were taken from this bucket (described later) and the bucket was covered with aluminum foil and left to develop and grow to the third larval stage for 10 days. After this time, larvae were collected using the Baermann technique and washed three times by repeated centrifugation for 15 minutes at 1,000 RPM and rinsing with tap water. Larvae were then placed in a petri dish of tap water with 0.250 ml Hank's Balanced Salt Solution (HBSS) containing 8 X 10^3 *S. neurona* sporocysts (collected from an adult male opossum and shown by western blot, polymerase chain reaction (PCR), and growth pattern in cell culture to be *S. neurona*). The petri dish was placed in a cool, dark environment for 5 days after which larvae were collected by centrifugation and washed two times with tap water. Larvae collected prior to sporocyst exposure were used as negative controls.

In order to test the ability of first and second stage strongyle larvae to ingest/attach to sporocysts, the four subsamples taken from the culture described above were placed in four separate petri dishes. Weights of the dry matter in each dish ranged from 66.6 g to 90.5 g. A solution containing 12 ml of Hank's Balanced Salt Solution with 6 X 10^5 *S. neurona* sporocysts was split into 3 ml aliquots and spread evenly over

the fecal matter in each petri dish. Dishes were covered in aluminum foil and placed in a cool dark environment for 10 days. After 10 days, larvae were collected from each sample using the Baermann technique and washed two times by repeated centrifugation for 15 minutes at 1,000 RPM and rinsing with tap water.

2.22 PCR:

Prior to DNA extraction, each sample was frozen with dry ice and then thawed with warm water 10X to help facilitate the extraction process. DNA was extracted from each sample with Qiagen DNeasy™ Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. PCR using oligonucleotide primers JNB25 and JD396 was used to determine the presence or absence of a 334 base pair product of *S. neurona* DNA in each sample.[2] Briefly, PCR was performed in standard reaction conditions (50 nM KCL, 10 mM tris-HCl, pH 9.0 at 25 °C. 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate) with a GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, CA) thermocycler with the following conditions: 72 °C, 3 min; 35 cycles of (94 °C, 30 sec; 37 °C, 1 min; 72 °C, 45 sec); 72 °C, 6 min; 4 °C, hold. PCR products were electrophoresed, visualized, and compared on a 1.8% agarose gel with ethidium bromide staining. These samples were tested on two separate occasions. A positive *Sarcocystis neurona* control was run along with the sample wells.

2.3 RESULTS:

A *Sarcocystis* PCR product was not detected in DNA extracted from any of the *Strongyle* groups. Positive controls were positive, untreated *Strongyle* larvae did not produce a *Sarcocystis* band.

2.4 DISCUSSION:

Strongyle L_1 and L_2 larvae feed in their environment prior to being ingested by the horse. It is reasonable to think that if *S. neurona* sporocysts were ingested by these larvae, it would occur during these phases of the lifecycle. Similar situations occur in nature. For example, *Anoplocephala perfoliata*, the equine tapeworm, infects horses after being ingested by the oribatid mite.

In this experiment, *S. neurona* DNA was not detected by PCR, indicating that the parasite was not present or present at levels below detection within the larvae. It is unlikely, but possible, that a small number of sporocysts could have gone undetected. The PCR technique used in this study has been previously found to be very sensitive and able to detect a DNA product of *S. neurona* with as little as 10 merozoites per 0.5 g of tissue (Keith Nelson, personal communication, 2001) making sporozoites unlikely to miss. There is the possibility that in the extraction process we were unable to excyst sporocysts within larvae and these went undetected. However, freezing and thawing prior to extraction decreases the chance of this occurrence.

Because this study did not take place under natural conditions (i.e. in an outdoor environment) the possibility cannot be ruled out that these larvae did not feed as they would in the soil. However, it is also unlikely that the larvae would come into contact with as large a number of sporocysts as were used in this study, so if ingestion does occur in nature, we should have been able to recreate a similar scenario *in vitro*.

Third-stage *Strongyle* larvae are covered with a protective sheath and are the stage of the parasite that penetrates the GI tract. It is possible that *S. neurona* sporocysts become entrapped in the sheath or become mechanically attached to this stage of the

parasite due to surface interactions and penetrate the GI tract along with the larvae. In this study, *S. neurona* DNA was not detected by PCR in third-stage larvae exposed to sporocysts for a 5-day period. This indicates that attachment either does not occur at this stage, does not occur in a liquid medium, or a longer period of exposure is needed for attachment. GI transit time in the horse does not exceed 5 days, making longer exposure times *in vivo* highly unlikely.

2.5 CONCLUSIONS:

We found no evidence from *in vitro* studies to support the hypothesis that *Strongyle* larvae can ingest or attract *S. neurona* sporocysts to transport them across the GI tract. This discredits the theory that Strongid®-C decreases the incidence of clinical EPM by an indirect method (i.e. decreasing the number of available transport hosts).

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CHAPTER 3

THE EFFECTS OF PYRANTEL TARTRATE ON *SARCOCYSTIS NEURONA* MEROZOITE VIABILITY

3.1 ABSTRACT:

Sarcocystis neurona is the etiological agent of equine protozoal myeloencephalitis (EPM), a neurological disease of horses. In this study, we tested the hypothesis that the anthelmintic pyrantel tartrate can kill *S. neurona* merozoites growing in equine dermal cell culture. *S. neurona* merozoites were exposed to a range of concentrations of pyrantel tartrate or sodium tartrate between 1 mM and 10 mM. Merozoites were then placed onto equine dermal cell cultures and incubated for two weeks to check for viability. At one and two weeks post inoculation, plaques were counted and the counts compared between treatment groups and controls using the Wilcoxon rank-sum test. Merozoites exposed to concentrations of pyrantel tartrate higher than 2.5 mM ($8.91X10^{-4}$ g/ml) did not produce plaques in equine dermal cells while those exposed to similar concentrations of the tartrate salt or medium alone produced significant numbers of plaques. These results demonstrate that pyrantel tartrate has activity against *S. neurona* merozoites *in vitro* and suggest that it may have activity against the sporozoite stage of the parasite found in the horse's intestinal tract.

3.2 INTRODUCTION:

Sarcocystis neurona is the etiological agent of equine protozoal myeloencephalitis (EPM), a neurological disease of horses.[1] Disease results from infection of the central nervous system (CNS) of equids with this protozoan parasite.[1] The lifecycle of *S. neurona* is complex and requires two hosts. The parasite replicates asexually in an intermediate host, forming tissue cysts in muscle that are infective to a carnivorous definitive host after ingestion. The nine-banded armadillo (*Dasypus novemcinctus*) has been found to be a natural intermediate host for *S. neurona*.[2,3] Cats have also been shown to act as intermediate hosts when experimentally infected with large numbers of *S. neurona* sporocysts.[4] A number of other intermediate hosts have been discovered recently and were described in Chapter One. Sexual replication of *S. neurona* occurs in the intestine of the definitive host, the opossum (*Didelphis virginiana*).[5] Infective sporocysts are shed into the environment in the feces of the opossum where they are consumed by an appropriate intermediate host to continue the life cycle.[6] Horses and ponies become infected when they ingest sporocysts from opossum feces, but *S. neurona* does not form tissue cysts in these species. Thus, equids are aberrant hosts.[1] It is likely that sporozoites are excysted from sporocysts in the digestive tract of the horse, penetrate the gut wall, and travel to the CNS of some horses where they are found as merozoites. The merozoite stage of the parasite replicates asexually in the neurons of the brain and spinal cord in equids, leading to the clinical signs of EPM.[7,8,9]

EPM is considered to be the most important protozoal disease of horses in the Americas.[10] Exposure to *S. neurona* is high, with a seroprevalence in horses in some areas exceeding 50%.[11,12,13] This high exposure rate, in addition to the seriousness of the

disease, supports the need for prevention, rather than treatment, as a method of controlling the disease. Daily feeding of an effective pharmaceutical preventative would be a possible means of protecting horses from infection with *S. neurona* and subsequent development of EPM. It has been speculated that pyrantel tartrate, the active ingredient of Strongid® C (Pfizer, Inc., New York City, NY), may have activity against *S. neurona*. Strongid® C is a daily anthelmintic feed supplement used to protect horses from strongyle larvae. Theoretically, daily pyrantel tartrate administration could prevent *S. neurona* infection in equids by killing sporozoites as they are excysted in the gut.

The purpose of this study was to determine if pyrantel tartrate has activity against *Sarcocystis neurona* merozoites. The merozoite stage of the parasite used in this study replicates in the central nervous system of horses and is not likely to be exposed to pyrantel tartrate, a drug that has low absorption and remains primarily within the digestive tract. However, it is believed that results attained from merozoite studies may be useful to model how the drug will work against the sporozoite stage of *S. neurona* found in the digestive tract. Positive results would lead to consideration of further studies investigating the possibility of using Strongid® C as a preventative drug for EPM in horses.

3.3 MATERIALS AND METHODS:

3.31 Preparation of Merozoites:

S. neurona merozoites (MI horse #1)[14] were grown and maintained on low passage (1-19) equine dermal cells (American Type Culture Collection CCL57, strain NBL-6) with Dulbecco's modified Eagle's medium (GIBCO, Rockville, MD) supplemented with 6% heat-inactivated fetal bovine serum, penicillin (100 U/ml), amikacin (100μg/ml), and amphotericin B (1.25 μg/ml) (solution hereafter referred to as DMEM). Medium containing asexually replicating stages of the parasite was removed from 4 heavily infected flasks (~100 ml). This solution was not filtered and, therefore, contained both the free merozoite stage of the parasite and any free equine dermal cells containing other asexually replicating stages of the parasite (early and late schizonts). The solution was placed in two 50 ml conical tubes and centrifuged for 40 minutes at 209 x *g*. After centrifugation, the supernatant was removed, and the pellet from each tube was resuspended in 1.5 ml of DMEM. The suspension from each tube was combined and stirred to evenly mix the parasite. Merozoites from five ~50 μl subsamples of this solution were counted using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA). The mean and standard deviation of these counts were determined. The merozoite count of the stock solution was $2.77 \pm 0.13 \times 10^6$ merozoites per ml. A 200 μl aliquot of this solution was placed in each of twelve 15 ml conical tubes making the final number of merozoites per tube approximately 5.54×10^5.

3.32 Cell Culture Preparation:

The aforementioned equine dermal cells (passage 19) were grown to confluency as determined by visual examination using an Olympus CK2 inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan) in eight 6-well cell culture plates (Corning, Inc., Corning, NY).

3.33 Preparation of Drug:

The activity of two chemical compounds was tested against *S. neurona* merozoites and all cellular stages of the asexually replicating parasite. Pyrantel tartrate (Sigma, Inc., St. Louis, MO) was tested as a possible preventative drug for EPM, and sodium tartrate (Sigma, Inc., St. Louis, MO) was used as a salt control. A 100 mM solution ($3.564X10^{-2}$ g/ml) of pyrantel tartrate (FW 356.4) in DMEM (adjusted to pH 7.98 with 5.0 N NaOH) was filtered through a 0.22 μm filter to remove contaminants and diluted in DMEM to concentrations of 10 mM, 7.5 mM, 5 mM, 2.5 mM, and 1 mM. A 100 mM solution ($2.30X10^{-2}$ g/ml) of sodium tartrate (FW 230.1) and DMEM (solution pH 7.78) was filtered through a 0.22 μm filter and diluted as above. For each drug, 1.0 ml of each dilution was added to a 15 ml conical tube containing merozoites (see above). One ml of DMEM alone (pH 7.88) was added back to two additional tubes as a negative control.

3.34 Experimental Design:

Tubes prepared above (see 3.33) were incubated at 37°C in 5% CO_2 for 24 hours. After this time, the tubes were centrifuged for 10 minutes at 209 x *g*, the supernatant

carefully removed to prevent pellet disruption, and 1.0 ml of DMEM added back to each tube as a washing step. Tubes were again centrifuged. This washing procedure was repeated twice to remove any residual drug from the pellet. After the final wash, the supernatant was carefully removed and the pellet gently resuspended in 4 ml of DMEM. The suspension from each tube was distributed between four wells (1.0 ml per well) of 6-well cell culture plates with confluent equine dermal cells. An additional 2 ml of DMEM was added to each well. The plates were incubated at 37°C in 5%CO_2 for 24 hours, after which all media was aspirated from all wells. Two ml of fresh DMEM was added back to each well and aspirated as a washing step. Three ml of DMEM was then added to maintain the cultures. DMEM in all wells was changed weekly for the duration of the experiment.

3.35 Plaque Number Determination:

After one week, equine dermal cell monolayers were observed for plaques and the plaque numbers counted with the inverted microscope (4X magnification). Wells were scanned in a uniform pattern from side to side in such a way that no fields overlapped and no plaque was counted twice. Plaque numbers for each pyrantel tartrate and sodium tartrate dilution (4 wells per dilution) were compared to numbers from the DMEM control (4 wells) using the Wilcoxon rank-sum test (exact test, two-sided). Plaque numbers from each sodium tartrate dilution (4 wells per dilution) were also compared to numbers from the DMEM control (4 wells) using the same statistical test. In addition, for each pyrantel tartrate dilution plaque numbers were compared to those of the sodium tartrate dilutions using the Wilcoxon rank-sums test (exact test, two-sided). A P-value of

$\leq$0.05 was used to determine significance for all comparisons. Plates were returned to the incubator after the first count, and plaques from each drug dilution were again counted one week later and analyzed using the same statistical methods.

3.36 DNA Extraction and PCR:

Cell culture supernatants and cell monolayers were both tested for the presence of *S. neurona* DNA using PCR. Medium was removed from all four wells of each drug dilution after two weeks and combined in 15 ml conical tubes (one tube per dilution). This media was centrifuged for 15 min at 1877 x *g*, the supernatant removed, and 0.5 ml of phosphate buffered saline (PBS) added back to the pellet. The solution from each tube was then transferred to separate 1.5 ml Eppendorf tubes and frozen at –20°C for later DNA extraction. One ml of alkaline chelating solution (ACS) was added back to the monolayer of cells in each well. Gentle rocking of the plate caused the monolayers to detach from the wells and become suspended in the ACS. The suspension from all four wells of each dilution was removed and combined as above. The suspensions were then centrifuged for 15 min at 1877 x *g*, the supernatants removed, and 0.5 ml of phosphate buffered saline (PBS) added back to the pellets. The suspensions from each tube were transferred to separate 1.5 ml Eppendorf tubes and frozen at –20°C for later DNA extraction.

Frozen samples were thawed in warm water and re-frozen with dry ice 3 times prior to extraction. DNA was extracted from each sample with Qiagen DNeasy™ Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

PCR, using oligonucleotide primers JNB25 and JD396, was used to determine the presence or absence of a 334 base pair product of *S. neurona* DNA in each sample.[15] Briefly, PCR was performed in standard reaction conditions (50 nM KCL, 10 mM tris-HCl, pH 9.0 at 25 °C. 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate) with a GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, CA) thermocycler with the following conditions: 72 °C, 3 min; 35 cycles of (94 °C, 30 sec; 37 °C, 1 min; 72 °C, 45 sec); 72 °C, 6 min; 4 °C, hold. PCR products were visualized and compared on a 1.8% agarose gel with ethidium bromide staining. These samples were tested on two separate occasions.

3.37 Drug Activity Control:

To ensure activity of the drug concentrations used, third-stage strongyle larvae were grown in vitro from equine feces containing large quantities of embryonated strongyle-type eggs. Larvae were collected after ten days of growth in a damp, room temperature, aerated environment using the Baermann technique, separated into six 15 ml conical tubes, and exposed to 1.0 ml solutions of pyrantel tartrate diluted with DMEM to concentrations of 0.0001 mM, 0.001 mM, 0.01 mM, 1mM, and 10 mM. One ml of DMEM was added to one tube of larvae as a negative control. After incubation at room temperature for 24 hours, larvae in each tube were assessed for viability based on motility using a dissecting microscope. Larvae were prodded with a drawn glass Pasteur pipette to distinguish non-motile from non-viable larvae. Larvae that did not move when prodded with the pipette were considered to be non-viable, and motile larvae viable. The number of non-viable larvae for each drug dilution was compared to the number of non-

viable larvae in the DMEM treated group using the Fisher's exact test. A P-value of ≤ 0.05 was used as the maximum critical value for evaluating the results.

3.4 RESULTS:

3.41 Plaque Numbers:

All pyrantel tartrate dilutions produced significantly lower plaque numbers when compared to the DMEM control after one and two weeks ($P \leq 0.03$) (Table 3.1). Merozoites exposed to pyrantel tartrate concentrations of 5 mM, 7.5 mM, and 10 mM produced no plaques after one and two weeks. Merozoites exposed to pyrantel tartrate at the concentration of 2.5 mM produced no plaques after one week and only a small number of plaques after two weeks. Merozoites exposed to pyrantel tartrate at the concentration of 1 mM produced plaques, however the number of plaques was significantly lower than that of the media control ($P \leq 0.03$) (Table 3.1). Plaque numbers for all sodium tartrate dilutions were not significantly different from the DMEM control after one and two weeks ($P > 0.05$) (Table 3.2).

All pyrantel tartrate dilutions produced significantly lower numbers of plaques than sodium tartrate dilutions of similar molarities ($P \leq 0.03$) with the exception of 1 mM after two weeks ($P > 0.03$) (Figures 3.1, 3.2; Table 3.3). DMEM control group plaque numbers for the sodium tartrate experiment and pyrantel tartrate experiment were not statistically different after one and two weeks ($P > 0.05$) (Table 3.3).

3.42 PCR:

A strong *Sarcocystis* PCR product was only detected in medium removed from the lowest concentration of pyrantel tartrate (1 mM). A weak *Sarcocystis* PCR product was detected in medium removed from pyrantel tartrate concentrations of 2.5 mM and greater. In contrast, a strong *Sarcocystis* PCR product was detected in the medium of all

sodium tartrate treatment groups and DMEM controls (Figure 3.3). Identical results were obtained with PCR of cells removed from culture wells (data not shown). Although a second PCR product of smaller size was seen in several wells, this product amplifies from equine dermal cell DNA, is not related, and should be disregarded in the interpretation of the gel (Figure 3.3).[14]

3.43 Drug Activity Control:

The number of viable third-stage strongyle larvae significantly decreased with exposure to all concentrations of pyrantel tartrate when compared to that of the DMEM control ($P < 0.0001$) (Table 3.4).

3.5 DISCUSSION:

In this study, the free merozoite stage of *S. neurona* and equine dermal cells containing asexually replicating stages of *S. neurona* (early and late schzonts) were exposed to various concentrations of pyrantel tartrate for 24 hours to determine if the drug had activity against the parasite. It was found that merozoites exposed to concentrations of pyrantel tartrate greater than 2.5 mM showed no growth when added to cell cultures. Pyrantel tartrate appears to be 100% lethal to merozoites at these concentrations. When merozoites were exposed to pyrantel tartrate at the concentration of 2.5 mM, no growth occurred after one week in culture, and limited growth was seen after two weeks. The drug at this concentration appeared to inactivate a large proportion of merozoites and may still be effective as a preventative.

Despite the absence of plaques with pyrantel tartrate concentrations greater than 2.5 mM, a faint *Sarcocystis* PCR product was detected in medium and cells from these wells. The PCR technique used in this study had been previously found to be quite sensitive: a DNA product can be detected with as little as 10 merozoites per 0.5 g of tissue (Keith Nelson, personal communication). The weak bands produced from PCR of medium and cells removed from wells with zero or few plaques may be due to small amounts of residual DNA from non-viable organisms trapped in the equine dermal cell monolayer. The fact that PCR products from wells with large plaque numbers showed a greater amplification of DNA indicated a larger amount of *S. neurona* DNA was present in those cultures due to parasite growth and replication. It is unlikely that viable organism remained in wells with no plaque growth; however, we are unable to

distinguish whether the remaining organisms causing the positive PCR reaction were viable or dead with this PCR technique.

All *S. neurona* merozoites exposed to sodium tartrate at molarities similar to that of pyrantel tartrate showed growth in culture similar to that of the DMEM control, and PCR detected a strong *Sarcocystis* PCR product in all treatment groups. These results lead to the conclusion that merozoite death was due to the presence of the tartrate compound and not due to the presence of DMEM alone or the tartrate salt alone. Additionally, the decrease in viability of third-stage strongyle larvae after exposure to concentrations of pyrantel tartate at and below that which inactivated merozoites shows that the drug was active at all concentrations used in this study.

The statistical comparison of the number of plaques produced in pyrantel tartrate dilutions and sodium tartrate dilutions indicates that in all groups, with the exception of 1 mM at two weeks, the number of plaques produced by the sodium tartrate dilutions was significantly greater than that of the pyrantel tartrate dilutions. The 1 mM pyrantel tartrate dilution produced a significantly lower number of plaques than the DMEM control; however, this group produced a much larger number of plaques than the numbers found at higher concentrations of pyrantel tartrate. This indicates that at the dose of 1 mM, the lowest dose tested in this study, pyrantel tartrate may not be effective against *S. neurona* merozoites.

The bioequivalence of Strongid® C and generic pyrantel tartrate against gastrointestinal parasites in horses has been previously demonstrated.[16] The concentration of pyrantel tartrate given daily to equids in the form of Strongid® C is 1.2 mg per lb. of body weight making the daily dose 1.2 g for a 1000 lb. horse. This

concentration greatly exceeds the amount of pyrantel tartrate found to kill merozoites in this study on a gram for gram basis, further evidence supporting the possibility that Strongid® C could be used as a preventative for EPM. However, drug dilution and distribution in the gastrointestinal tract of the horse may vary, making it difficult to compare an *in vivo* gram per volume dose to the dosage tested in this study. Pyrantel tartrate was found to be ineffective against *S. neurona* infection in gamma-interferon gene knockout mice[17]; however, the digestive tracts and gastrointestinal transit times of mice and equids vary greatly. Future *in vivo* studies will be conducted to determine if Strongid® C protects horses from infection with *S. neurona.*

Pyrantel tartrate is an anthelminthic compound that acts in two ways: 1) it possesses nicotine-like properties and acts similarly to acetylcholine (ACh), and 2) it inhibits acetylcholinesterase. [18] These properties make the drug a depolarizing neuromuscular blocking agent in susceptible parasites. Exposure to pyrantel tartrate leads to paralysis of the susceptible organism.[18] The presence of ACh has been detected in bacteria and primitive organisms, such as the blue-green algae, yeast, fungi, tubellaria, protozoa (*Trypanosoma rhodesiense*, *Paramecium*), nematodes, and sponges.[19,20] In studied organisms, ACh occurs in both neuronal and non-neuronal tissues. Non-neuronal ACh appears to be involved in the regulation of basic cell functions, such as proliferation, differentiation, cell-cell contact, immune functions, secretion, and absorption. [20]It has been speculated that ACh may be involved in the rapidly moving protozoa like trypanosomes but not in sluggish ameboid movement.[21] The fact that ACh has been found in a wide variety of organisms leads us to consider the possibility that *S. neurona* may also synthesize ACh. If ACh is present in *S. neurona*, pyrantel tartrate could affect

the parasite by disrupting basic cell functions regulated by ACh or by disrupting the movement of *S. neurona* related to the ACh molecule. The presence and possible roles of ACh in *S. neurona* need to be thoroughly explored before any conclusions can be made about the mechanism of action of pyrantel tartrate in this organism.

The merozoite stage of *S. neurona* is found in the brain and spinal cord of horses and is not exposed to pyrantel tartrate, a drug that remains primarily in the digestive tract. However, because the merozoite stage has some similarities to the sporozoite stage found in the gut of the horse and is readily available for *in vitro* studies, we elected to initially investigate the effects of pyrantel tartrate on this stage of the parasite. The fact that pyrantel tartrate appears to kill both free merozoites and stages of *S. neurona* asexually replicating within cells is encouraging and provides further evidence that the drug may be able to kill more than one stage of the parasite.

3.6 CONCLUSION:

Pyrantel tartrate at concentrations greater than 2.5 mM ($8.91X10^{-4}$ g/ml) kills both free *Sarcocystis neurona* merozoites and stages of *S. neurona* asexually replicating within equine dermal cells. This result has prompted further investigations into the possibility of using Strongid® C as a preventative for EPM in horses.

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Treatment Group	Plaque Numbers (mean)		Standard Deviation		P value*	
	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2
DMEM	86.75	143.25	7.09	7.62	---	---
0.001M	64.50	120.50	8.06	8.96	0.03	0.03
0.0025M	0	5.25	0	2.63	0.03	0.03
0.005M	0	0	0	0	0.03	0.03
0.0075M	0	0	0	0	0.03	0.03
0.01M	0	0	0	0	0.03	0.03

Table 3.1. Plaque numbers for pyrantel tartrate concentrations. *P-value refers to comparison between pyrantel tartrate dilutions and the DMEM control (Wilcoxan rank sum test, two tailed, exact test).

Treatment Group	Plaque Numbers (mean)		Standard Deviation		P value*	
	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2
DMEM	89.75	141.25	3.10	20.0	---	---
0.001M	96.25	133.00	10.8	13.0	0.49	0.69
0.0025M	82.25	140.50	11.4	18.2	0.69	0.97
0.005M	93.50	137.75	1.92	14.0	0.14	0.89
0.0075M	94.50	135.00	4.20	15.5	0.14	0.69
0.01M	79.50	119.75	7.33	21.0	0.06	0.20

Table 3.2. Plaque numbers for sodium tartrate concentrations. *P-value refers to comparison between sodium tartrate dilutions and the DMEM control (Wilcoxan rank sum test, two tailed, exact test).

	Plaque Numbers— Week 1 (mean ± SD)		Plaque Numbers— Week 2 (mean ± SD)		P value*	
	ST	PT	ST	PT	Week 1	Week 2
DMEM	89.75±3.10	86.75±7.1	141.3±20.0	143.25±7.6	0.31	0.68
0.001M	96.25±10.8	64.50±8.1	133±13.0	120.50±9.0	0.03	0.20
0.0025M	82.25±11.4	0.00±0	140.5±18.2	5.25±2.6	0.03	0.03
0.005M	93.50±1.92	0±0	137.8±14.0	0±0	0.03	0.03
0.0075M	94.50±4.20	0±0	135±15.5	0±0	0.03	0.03
0.01M	79.50±7.33	0±0	119.8±21.0	0±0	0.03	0.03

Table 3.3. Plaque numbers for sodium tartrate (ST) and pyrantel tartrate (PT) concentrations. *P value refers to comparison between sodium tartrate and pyrantel tartrate numbers at the same molarities (Wilcoxan rank sum test, two tailed, exact test).

Treatment Group	Number of Viable Larvae Before Pyrantel Exposure	Number of Viable Larvae After Pyrantel Exposure	Number of Non-Viable Larvae After Pyrantel Exposure	Percent Non-Viable	P-value*
DMEM	8	7	1	12.5	---
0.000001M	18	1	17	94.4	< 0.0001
0.00001M	13	0	13	100	< 0.0001
0.0001M	16	0	16	100	< 0.0001
0.001M	27	1	26	96.3	< 0.0001
0.01M	14	0	14	100	< 0.0001

Table 3.4. Numbers of viable and non-viable strongyle larvae after exposure to pyrantel tartrate. *P value refers to comparisons between non-viable larvae from pyrantel tartrate dilutions and the DMEM control (Fisher's exact test, 2-tailed).

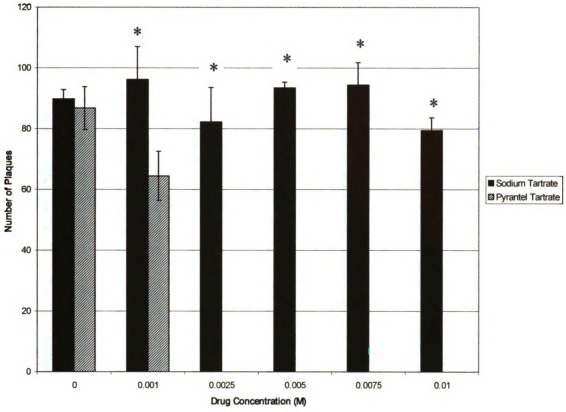

Figure 3.1. Plaque numbers (mean of four wells) one week after culture inoculation. (*) indicates a significant difference between plaque numbers in sodium tartrate treatment groups and pyrantel tartrate treatment groups. I = one standard deviation.

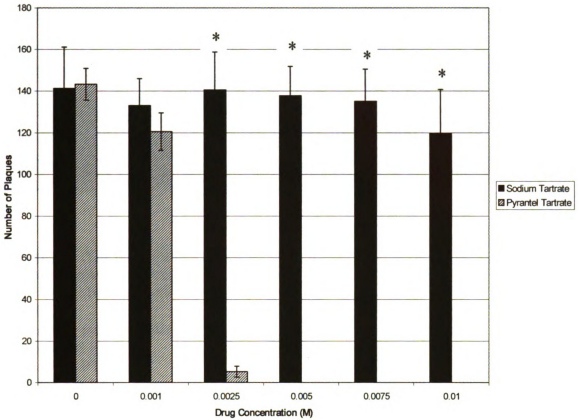

Figure 3.2. Plaque numbers (mean of four wells) two weeks after culture inoculation. (*) indicates a significant difference between plaque numbers in sodium tartrate treatment groups and pyrantel tartrate treatment groups. I = one standard deviation.

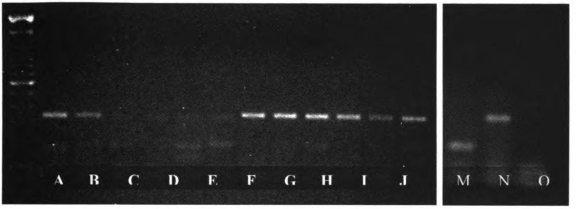

Figure 3.3. PCR of media removed from cell cultures infected with merozoites treated with the following solutions:
Lane 1) 100 bp size standard
A) DMEM
B) 0.001M pyrantel tartrate (PT)
C) 0.0025M PT
D) 0.005M PT
E) 0.0075M PT
F) 0.01M PT
G) DMEM
H) 0.001M sodium tartrate (ST)
I) 0.0025M ST
J) 0.005M ST
K) 0.0075M ST
L) 0.01M ST
M) Equine dermal cell DNA control
N) *S. neurona* DNA (+ control)
O) PCR solution only (- control)

CHAPTER 4

THE EFFECTS OF PYRANTEL TARTRATE ON *SARCOCYSTIS NEURONA* SPOROZOITES IN CELL CULTURE AND IN GAMMA INTERFERON KNOCK-OUT MICE

4.1 INTRODUCTION:

Equine protozoal myeloencephalitis (EPM), a neurologic disease of horses, is caused by the protozoan parasite *Sarcocystis neurona*[1]. The lifecycle of *S. neurona* is complex and involves the definitive host, the opossum (*Didelphis virginiana*)[2], and a variety of intermediate hosts including the nine-banded armadillo (*Dasypus novemcinctus*)[3,4], the striped skunk (*Mephitis mephitis*)[5], the raccoon (*Procyon lotor*)[6], and the domestic cat (*Felis domesticus*)[7]. Horses are considered aberrant hosts and become infected with *S. neurona* after accidentally ingesting the sporocyst stage of the parasite passed in opossum feces. It is hypothesized that sporozoites contained within the sporocyst are excysted in the gastrointestinal tract of the horse, then penetrate the gut wall and enter the bloodstream[8]. From the bloodstream they travel to the central nervous system and replicate asexually in the brain and spinal cord as merozoites leading to the clinical signs of EPM.

Exposure to *S. neurona* is high, with the seroprevalence in horses in some areas exceeding 50%[9]. However, the number of horses with clinical signs of disease is much lower. This high exposure rate, combined with the serious consequences of the disease, indicates a form of prevention, rather than treatment, would be a logical way of controlling disease. Pyrantel tartrate at concentrations greater than 2.5 mM has been found to be lethal to *S. neurona* merozoites in cell culture[10]. This drug is available in a pelleted form, Strongid®-C (Pfizer, Inc., New York, NY), that is fed daily to horses to help prevent Strongyle infection. If pyrantel tartrate were also found to be effective

against the sporozoite stage of the parasite, daily pyrantel administration could theoretically prevent *S. neurona* infection by killing sporozoites as they are excysted in the GI tract.

The purpose of the present study was to determine if pyrantel tartrate has efficacy against the sporozoite stage of *S. neurona* in either cell culture or in a gamma interferon knock-out mouse model of EPM[11].

4.2 MATERIALS AND METHODS: CELL CULTURE:

4.21 Cell culture:

Low passage (p22) equine dermal cells (American Type Culture Collection CCL57, strain NBL-6) were grown to confluency as determined by visual examination using an Olympus CK2 inverted microscope in four, 6-well cell culture plates (Corning, Inc., Corning, NY).

4.22 Sporocysts:

Sporocysts were harvested from intestines of laboratory reared, specific pathogen free opossums, which were euthanized 14 days after oral inoculation with *Sarcocystis neurona*-infected cat tissue[7]. Sporocysts were cleaned and stored using the technique described by Murphy and Mansfield[12].

4.23 Preparation of sporozoites:

Approximately 100,000 *S. neurona* sporozoites were excysted using methods described previously[12]. Excysted sporozoites were confirmed present by visually assessing a sample of the solution under an Olympus CK2 inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Solution containing intact sporocysts, remnants of cysts, and sporozoites was placed onto a 60% Percoll™ (Amersham Biosciences, Piscataway, NJ) and phosphate buffered saline (PBS) gradient. An additional tube containing the same 60% Percoll™ and PBS solution plus Percoll™ density marker beads and the tube containing sporocysts and sporozoites were centrifuged for 60 minutes at 12,000 RPM. After this time the sporozoite solution below the density marker bead line

representing 1.05 g/ml was removed and added to 30 ml of DMEM in a 50 ml conical tube. This tube was centrifuged for 30 minutes at 2,000 RPM as a washing step. The supernatant was removed and 1.2 ml DMEM added back to the pellet. This solution was split evenly between six, 15 ml conical tubes.

4.24 Preparation of drug for use in cell culture:

A 100 mM solution ($3.564X10^{-2}$ g/ml) of pyrantel tartrate (FW 356.4) in DMEM (Dulbecco's modified Eagle's medium (GIBCO, Rockville, MD) supplemented with 6% heat-inactivated fetal bovine serum, penicillin (100 U/ml), amikacin (100μg/ml), and amphotericin B (1.25 μg/ml) was pH adjusted with 5.0 N NaOH, filtered through a 0.22 μm filter to remove contaminants, and diluted in DMEM to concentrations of 10 mM, 5 mM, 1 mM, 0.5 mM, and 0.1 mM. One ml of each dilution was added to a 15 ml conical tube containing sporozoites (see 4.23). One ml of DMEM alone was added back to one additional tube as a negative control.

4.25 Experimental procedure:

Tubes prepared above were incubated at 37°C in 5% CO_2 for 24 hours. After this time, the tubes were centrifuged for 20 minutes at 209 x *g*, the supernatant carefully removed to prevent pellet disruption, and 1.0 ml of DMEM added back to each tube as a washing step. Tubes were centrifuged in the same manner. This washing procedure was repeated twice to remove any residual drug from the pellet. After the final wash, the supernatant was carefully removed and the pellet gently resuspended in 2 ml of DMEM. The suspension from each tube was distributed between four wells (0.5 ml per well) of 6-

well cell culture plates with confluent equine dermal cells. An additional 2.5 ml of DMEM was added to each well. The plates were incubated at 37°C in 5% CO_2 for three weeks with weekly media changes. Plates were observed weekly for plaque growth with an Olympus CK2 inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

4.26 Plaque Number Determination:

After three and four weeks, equine dermal cell monolayers were observed for plaques, and the plaque numbers counted with the microscope described previously (see 4.21). Wells were scanned in a uniform pattern from side to side in such a way that no fields overlapped and no plaque was counted twice.

4.27 Statistical analysis:

Plaque numbers for each pyrantel tartrate dilution (4 wells per dilution) were compared to numbers from the DMEM control (4 wells) using the Wilcoxon rank-sum test (exact test, two-sided). A P-value of ≤ 0.05 was used to determine significance for all comparisons. Plates were returned to the incubator after the first count, and plaques from each drug dilution were again counted one week later and analyzed using the same statistical methods.

4.3 MATERIALS AND METHODS: MICE:

4.31 Mice:

Fifteen female, 12 week old, IFN-γ-knockout mice (BALB/c-Ifng^{tm1Ts}) were acquired from Jackson Laboratories, Bar Harbor, ME. Mice were housed in filtered cages in a laminar flow rack, fed sterilized food and water, and kept on autoclaved bedding. All mice weighed approximately 20 grams.

4.32 Sporocysts:

Sporocysts were harvested from intestines of laboratory reared, specific pathogen free opossums, which were euthanized 14 days after oral inoculation with *Sarcocystis neurona* strain SN-37R raccoon tissue[6]. Sporocysts were cleaned and stored using the technique described by Murphy and Mansfield[12].

4.33 Experimental procedure:

Mice were randomly assigned to three treatment groups of 5 mice each. Mice in Group 1 were dosed once daily for six days with 0.2 ml sterilized distilled water through a 5 French rubber urinary catheter. On day 3 of dosing, these mice were given 1000 *S. neurona* sporocysts via a 22 ga gavage needle. Mice in Group 2 were dosed once daily for six days with 0.2 ml of 5 mM pyrantel tartrate (Sigma, Inc., St. Louis, MO, FW 356.4) solution in sterilized distilled water. On day 3 of dosing, mice in this group received 1000 *S. neurona* sporocysts via a 22 ga gavage needle. Mice in Group 3 received six days of 0.2ml 5 mM pyrantel tartrate solution in distilled water via a 5F rubber urinary catheter. Group 3 remained unchallenged.

Mice were observed daily for abnormal clinical signs. Any mouse unable to ambulate or consume food or water or in respiratory distress was euthanized with gradually increasing doses of carbon dioxide in an approved chamber according to the guidelines of the AVMA[13]. Mice in Group 3 were euthanized 14 days after the death of the last infected mouse. A complete necropsy was performed on each mouse, and samples of brain, liver, kidney, lung, and spleen were collected and fixed in 10% neutral buffered formalin. A sample of brain tissue from each mouse was also prepared for cell culture.

4.34 Histopathology:

Following fixation in formalin, tissues were dehydrated through a graded series of alcohol to xylol, embedded in paraffin, sectioned at 5 microns, and processed routinely. All tissue sections were stained with hematoxylin and eosin (HE), and were examined via light microscopy by a board-certified veterinary anatomic pathologist (JSP), who was blinded to treatment group.

4.35 Statistical analysis:

Mean ($\pm$ standard deviation) post survival time was compared between Group 1 and Group 2 using the student's t-test ($P \leq 0.05$).

4.4 RESULTS:

4.41 Cell culture:

Zero plaque growth was seen in the 5 mM and 10 mM pyrantel tartrate treated groups after 3 weeks of growth. Zero plaque growth was seen in the 10 mM treated group and minimal plaque growth was seen in the 5 mM treated group after 4 weeks of incubation. One mM, 5 mM, and 10 mM pyrantel tartrate treated groups had plaque numbers significantly lower than the media control ($P < 0.03$) after both 3 and 4 weeks of growth (Figure 4.1).

4.42 Mice:

Untreated mice (Group 1) survived a mean of 31.4 ± 2.3 days. Treated mice (Group 2) survived a mean of 35 ± 1.9 days. This was significant increase in mean survival time ($P=0.03$).

Additionally, 5/5 mice in Group 1 had evidence of both encephalitis and pneumonia when examined histopathologically while only one mouse in Group 2 had histologic lesions consistent with encephalitis and pneumonia. Three additional mice in this group showed lesions of pneumonia only, and one mouse had no histologic lesions. No protozoal organisms were seen in the brains of mice in Group 2 while 3/5 of the mice in Group 1 contained protozoa. Protozoa were also seen in the lungs of 1/5 mice in Group 1 and 3 out of the four mice with lesions in Group 2. Merozoites were cultured from the brain tissue of all five mice in Group 1 and all five mice in Group 2. Mice in Group 3 had no histologic lesions. No merozoites were cultured from any mice in Group 3.

4.5 DISCUSSION:

In this study, the action of pyrantel tartrate was tested against the sporozoite stage of *S. neurona* in two different ways. First, the effects of the drug directly on sporozoites were observed using a previously published cell culture drug screening technique[10]. The drug was found to be 100% lethal to sporozoites at pyrantel tartrate concentrations of 5 mM and higher. Pyrantel at the concentration of 1 mM also caused a significantly lower number of plaques than the media control. These concentrations are identical to those that have been shown to cause merozoite death in a previous study[10]. Results of this study show that pyrantel tartrate has a direct effect on sporozoite numbers and their ability to reproduce in cell culture.

The second part of this study tested the *in vivo* effects of pyrantel tartrate on *S. neurona* sporocysts using a mouse model. Mice treated with pyrantel prior to being dosed with sporocysts lived significantly longer than those that were dosed with sterile water. This indicates that the drug may decrease the number of infective sporozoites, making the number insufficient enough to penetrate the central nervous system (CNS) or small enough that they are able to be cleared by the host's immune system before they can cause CNS damage. This hypothesis is supported by a study completed by Dubey that established a time course of infection in gamma interferon knock-out mice to understand the pathogenesis of this parasite[14]. The study found that infection and replication occur in the lungs prior to the CNS.

4.6 CONCLUSIONS:

From this study we conclude that pyrantel tartrate is lethal to *S. neurona* sporozoites at concentrations of 5 mM and greater. The drug also affects sporozoite infection in gamma interferon knock-out mice causing treated mice to live a significantly longer duration of time following *S. neurona* sporozoite infection and to have different organs affected with similar histologic lesions than mice that did not receive pyrantel tartrate.

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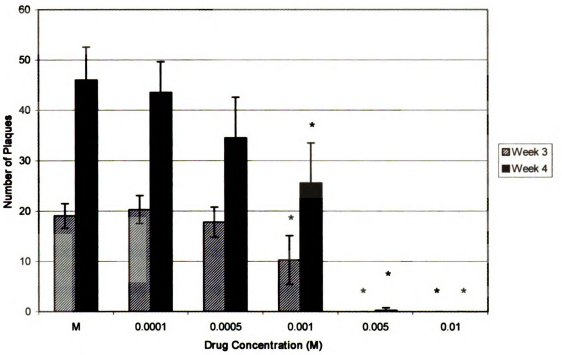

Figure 4.1. Pyrantel tartrate plaque assay. Plaque numbers (mean of four wells) three and four weeks after culture inoculation. (*) indicates a significant difference between plaque numbers in media control and pyrantel tartrate treatment groups. I = one standard deviation.

CHAPTER 5

SARCOCYSTIS NEURONA: IN VITRO EXPOSURE OF MEROZOITES TO ACETYLCHOLINE AND ATROPINE

5.1 ABSTRACT:

Sarcocystis neurona, a protozoan parasite, is the etiologic agent of equine protozoal myeloencephalitis (EPM), a neurological disease of horses. *In vitro* studies have shown that pyrantel tartrate, a depolarizing neuromuscular agent that works at acetylcholine (ACh) receptors, has activity against the merozoite stage of this parasite[1]. The following study was designed to test the hypothesis that *S. neurona* merozoites have ACh receptors. Using the drug screening assay described by Kruttlin et al.[1], the activity of acetylcholine chloride and atropine sulfate at various concentrations was tested against *S. neurona* merozoites. Additionally, monoclonal antibodies against the ACh receptor α protein and appropriate controls were obtained and used in Western blotting in an attempt to detect this receptor protein in merozoites. Merozoites exposed to ACh showed no decrease in viability after two weeks at all tested concentrations (0.001 mM to 10 mM) while those exposed to atropine showed a decrease in viability at the concentration of 10 mM. The ACh receptor α protein was not detected with Western blot.

Preliminary results obtained by the previously described assays indicate that *S. neurona* merozoites do not possess acetylcholine receptors and suggest that pyrantel tartrate acts at a different target site in this parasite; however, the question remains as to whether this assay is a valid technique for ACh screening in protozoa. Further studies using gas chromatography and other proven techniques must be performed to support described results.

5.2 INTRODUCTION:

Sarcocystis neurona is the etiologic agent of equine protozoal myeloencephalitis (EPM), a neurologic disease of horses[2]. The coccidian parasite uses the opossum (*Didelphis virginiana*) as a definitive host and a variety of mammals as intermediate hosts[4,5,6]. Sexual replication of *S. neurona* occurs in the intestine of the definitive host, after which infective sporocysts are shed into the environment to be consumed by an appropriate intermediate or aberrant host[6]. The likely pathogenesis is that sporocysts excyst releasing sporozoites the gastrointestinal tract of the horse that penetrate the gut wall and travel to the central nervous system where they replicate asexually in neurons as merozoites. The damage resulting from merozoite replication leads to the clinical signs of EPM[7].

Pyrantel tartrate is an anthelmintic compound that possesses nicotine-like properties, acting similar to acetylcholine (ACh) and by inhibiting acetylcholinesterase[8]. These properties make the drug a depolarizing neuromuscular-blocking agent in susceptible parasites. *In vitro* studies have found pyrantel tartrate to be lethal to *S. neurona* merozoites at concentrations greater than 2.5 mM (8.91×10^{-4} g/ml)[1]; however, the mechanism of action of the drug in this parasite is unknown. While the presence of ACh has not been found in *Sarcocystis* species specifically, it has been detected in several protozoal species (*Trypanosoma rhodesiense, Paramecium*), as well as bacteria, and primitive organisms such as blue-green algae, yeast, fungi, tubellaria, nematodes, and sponges[10]. Because ACh has been found in a wide variety of organisms and because pyrantel tartrate, a compound that acts similarly to ACh, causes lethality in merozoites, we hypothesized that *S. neurona* merozoites possess ACh receptors. If ACh receptors are

present in *S. neurona* merozoites, pyrantel tartrate would be likely to affect the parasite by disrupting basic cell functions regulated by ACh, as seen in *Giardia lamblia*[11], or by disrupting merozoite movement related to the ACh molecule, similar to that of helminth larvae. In these studies, ACh drug screens against merozoites and testing for the ACh receptor α protein showed no evidence that *S. neurona* merozoites have ACh receptors.

5.3 MATERIALS AND METHODS:

5.31 Preparation of Merozoites:

S. neurona merozoites (MI horse #1) were grown and maintained on low passage (1-19) equine dermal cells (American Type Culture Collection CCL57, strain NBL-6) with Dulbecco's modified Eagle's medium (GIBCO, Rockville, MD) supplemented with 6% heat-inactivated fetal bovine serum, penicillin (100 U/ml), amikacin (100μg/ml), and amphotericin B (1.25 μg/ml) (solution hereafter referred to as DMEM) as described previously[12]. Medium containing asexually replicating stages of the parasite was removed from 7 heavily infected flasks (~175 ml). This solution was not filtered and, therefore, contained both the free merozoite stage of the parasite and any free equine dermal cells containing other asexually replicating stages of the parasite (early and late schizonts). The solution was placed in a 250 ml flask and centrifuged for 90 minutes at 209 x *g*. After centrifugation, the supernatant was removed, and the pellet from the flask resuspended with DMEM and transferred to a 50 ml conical tube. The tube was centrifuged for 30 minutes at 209 x g. The supernatent was removed and 4 ml DMEM was added back to the pellet. Merozoites from four ~50 μl subsamples of this solution were counted using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA). The mean and standard deviation of these counts were determined. The merozoite count of the stock solution was $9.08 \pm 0.72 \times 10^6$ merozoites per ml. A 200 μl aliquot of this solution was placed in each of eighteen 15 ml conical tubes making the final number of merozoites per tube approximately 5.04×10^5.

5.32 Cell Culture Preparation:

Equine dermal cells described above (passage 25) were seeded in equal numbers and grown to confluency as determined by visual examination using an Olympus CK2 inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan) in twelve 6-well cell culture plates (Corning, Inc., Corning, NY).

5.33 Preparation of Drug:

The activity of acetylcholine chloride and atropine sulfate (Sigma, Inc., St. Louis, MO) was tested against *S. neurona* merozoites and all cellular stages of the asexually replicating parasite. A 0.1M solution ($1.81X10^{-2}$ g/ml) of ACh (FW 181) in DMEM was filtered through a 0.22 μm filter to remove contaminants and diluted in DMEM to concentrations of 10 mM, 1 mM, 0.1 mM, 0.01 mM, and 0.001 mM. A 0.1M solution ($6.76X10^{-2}$ g/ml) of sodium tartrate (FW 676) and DMEM was filtered through a 0.22 μm filter and diluted as above. For each drug, 1.0 ml of each dilution was added to a 15 ml conical tube containing merozoites (see above). One ml of DMEM alone was added back to two additional tubes as a negative control. Additionally, sodium sulfate (Sigma, Inc., St. Louis, MO) was tested as a sulfate control at concentrations identical to that of atropine sulfate. A 5 mM solution of pyrantel tartrate ((Sigma, Inc., St. Louis, MO, FW 356.4) in DMEM was also prepared and filtered as above.

5.34 Experimental Design:

Direct effects of ACh and atropine. Tubes prepared above were incubated at 37°C in 5% CO_2 for 24 hours. After this time, the tubes were centrifuged for 10 minutes at 209

x *g*, the supernatant carefully removed to prevent pellet disruption, and 1.0 ml of DMEM added back to each tube as a washing step. Tubes were again centrifuged. This washing procedure was repeated twice to remove any residual drug from the pellet. After the final wash, the supernatant was carefully removed and the pellet gently resuspended in 4 ml of DMEM. The suspension from each tube was distributed between four wells (1.0 ml per well) of 6-well cell culture plates with confluent equine dermal cells. An additional 2 ml of DMEM was added to each well. The plates were incubated at 37°C in 5%CO_2 for 24 hours, after which all media was aspirated from all wells. Two ml of fresh DMEM was added back to each well and aspirated as a washing step. Three ml of DMEM was then added to maintain the cultures. DMEM in all wells was changed weekly for the duration of the experiment.

Ability of atropine to block the activity of pyrantel tartrate. One ml of each atropine solutions of 10 mM, 1 mM, 0.1 mM, and 0.01 mM was added back to separate 15 ml conical tubes. One ml of DMEM was added to a fifth tube as a media control, and one ml of 5 mM pyrantel tartrate was added to a sixth tube as a drug activity control. Tubes prepared above were incubated at 37°C in 5% CO_2 for one hour. After this time, the tubes were centrifuged for 10 minutes at 209 x *g*, the supernatant carefully removed to prevent pellet disruption. One ml of 5 mM pyrantel tartrate was added to each tube with the exception of the DMEM control. Tubes were incubated at 37°C in 5% CO_2 for 24 hours and then treated as above.

3.35 Plaque Number Determination:

After one week, equine dermal cell monolayers were observed for plaques and the plaque numbers counted with the inverted microscope described previously (4X magnification). Wells were scanned in a uniform pattern from side to side in such a way that no fields overlapped and no plaque was counted twice. Plaque numbers for each ACh and atropine dilution (4 wells per dilution) were compared to numbers from the DMEM control (4 wells) using the Dunnett's t-test (two-sided). Plaque numbers from each atropine+pyrantel tartrate dilution (4 wells per dilution) were also compared to numbers from the DMEM control (4 wells) and pyrantel tartrate drug activity control using the same statistical test. A p-value of ≤ 0.05 was used to determine significance for all comparisons. Plates were returned to the incubator after the first count, and plaques from each drug dilution were again counted one week later and analyzed using the same statistical methods.

5.36 Western blot:

S. neurona merozoites were harvested from equine dermal cell culture, centrifuged, and the pellet denatured in sample buffer (0.5 M Tris (pH 7.4) with 10% sodium dodecyl sulfate, 20% glycerol and 5% β-mercaptoethanol) for 5 minutes at 95° C. Proteins were loaded into wells of a 10-well 4% stacking gel with and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 11-17% linear gradient gels. For reference, biotinylated broad range molecular weight size standard markers were run on each gel. Twenty microliters of AChR positive control (BC3H1 Lysate) (BD Transduction Laboratories, San Diego, CA), was loaded on the gel

as a positive control for Western blotting experiments. Separated proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes for 1.5 hours and blocked overnight in blocking buffer (1% bovine serum albumen in 0.5% Tween-Tris Buffered Saline (TTBS)). Blots were dried and frozen for later use.

For testing of samples, the blots were wetted in TTBS and clamped in a slot blot apparatus. AChR antibody described above was diluted at 1:200 with blocking buffer and 650µl placed on each of two wells run with positive control (BC3H1 Lysate) and each of 4 wells containing blotted proteins from *S. neurona* merozoites. Serum samples from *S. neurona* positive and negative horses were also added to two wells to serve as transfer controls for the *S. neurona* merozoite preparation. Detection of two antigens of approximately 30- and 16-kDa serves as the criterion for a positive test[13]. Solutions were left to incubate overnight. Wells were then washed thoroughly with TTBS, and 650µl Biotin-labeled anti-mouse (7.5 µl/ml BTTBS) was applied to all wells as the secondary conjugate for 4 hours. Wells were washed again and 650µl of ExtrAvidin® (Sigma, St. Louis, MO) added to each well for 45 minutes. After removal of ExtrAvidin® and washing with TTBS, the blot was developed in an aminoethyl carbazole substrate.

5.4 RESULTS:

5.41 Plaque numbers:

ACh concentrations of 1 mM and 10 mM had significantly decreased plaque numbers from the DMEM control after one week of incubation ($p < 0.05$). The ACH concentration of 10 mM had significantly decreased plaque numbers compared to control after 2 weeks of incubation ($p < 0.05$) (Table 5.1, Figure 5.1). All atropine dilutions, with the exception of 10 mM, produced plaque numbers that were not significantly different from the DMEM control after both one and two weeks of growth (Table 5.1, Figure 5.2). Atropine at the concentration of 10 mM caused a significant decrease in plaque numbers in both week 1 and week 2 ($p < 0.05$).

Merozoites exposed to 5 mM pyrantel tartrate alone produced zero plaques after both one and two weeks. Merozoites exposed first to atropine sulfate and then to pyrantel tartrate produced zero plaques at all atropine concentrations. DMEM control for these plates produced plaque growth similar to that of the DMEM controls for the ACh and atropine experiments described above.

5.42 Western blot:

The Western blot results showed no evidence of an ACh receptor in *S. neurona.* Monoclonal antibodies against the ACh receptor α protein did not produce a detectable band when incubated with *S. neurona* merozoite proteins. A 49 kDa band was detected with the BC3H1 Lysate positive control. The positive blot control produced bands at 30- and 16-kDa as expected. The negative blot control (*S. neurona* merozoites incubated with *S. neurona* negative horse sera) was negative.

5.5 DISCUSSION:

Pyrantel acts as an agonist of acetylcholine receptors of nematodes and works by depolarizing muscle membranes. While it has been shown to be lethal to *S. neurona* merozoites, the mechanism of action in this species has yet to be explained. One step in determining if the drug works by a mechanism similar to that in nematodes is to expose merozoites to ACh and determine if the effect is similar to that of pyrantel exposure. The ACh doses used in this study cause paralysis in parasitic helminth worms[14]; therefore rendering them unable to counteract host GI parastaltic movements and leading to their passage from the host GI tract. *S. neurona* merozoites rely on a circular "burrowing" movement to penetrate neurons and other cells, and paralysis from ACh or pyrantel exposure could block their ability to enter cells and, therefore, stop merozoite replication and plaque growth. Pyrantel pamoate has been found to affect *Giardia lamblia* trophozoites by decreasing flagellar beating frequency and by causing severe changes in the cytoplasm and peripheral vesicles[15]. While merozoites do not possess flagella, it is possible that cytoplasmic or vesicle changes, similar to those seen in *Giardia* sp., could occur in merozoites after exposure to pyrantel tartrate.

Exposure to a variety of concentrations of acetylcholine chloride did not decrease plaque growth or affect *S. neurona* merozoite replication after two weeks of growth in this study. If *S. neurona* merozoites possess ACh receptors, the application of ACh at the concentrations in this study should theoretically have caused overstimulation of the receptors and paralysis of the parasite or a similar observable phenomenon. The absence of a significant decrease in plaque growth after two weeks of growth seen with exposure to ACh may indicate that *S. neurona* merozoites do not possess ACh receptors and that

pyrantel tartrate works at a different target site in this parasite. However, it cannot be ruled out that the lack of change in viability after ACh exposure could have been a result of ACh breakdown in solution over time or to parasite recovery prior to entering equine dermal cells. A chloride control was not deemed necessary for this series of experiments because the ion is found in DMEM media and has not previously affected *S. neurona* viability. Additionally, completion of an experiment exposing Strongyle larvae to ACh at concentrations used in this study and run parallel with merozoite exposure would have strengthened the argument that merozoites do not possess the receptor. However, it may also be possible that concentrations of ACh higher than those that affect parasitic helminths may be needed to change the viability of *S. neurona* merozoites. Nevertheless, in separate laboratory experiments we have found ACh concentrations of up to 100 mM have been unable to cause a significant decrease in *S. neurona* induced plaque numbers.

In helminth worms, atropine is an antagonist at ACh receptors and stimulates motility and contraction[14]. If *S. neurona* merozoites have ACh receptors it would be logical to expect similar changes in motility followed by the inability of the parasite to penetrate host cells and eventual death of the parasite. If the ACh molecule itself is vital to merozoite survival and ACh receptors are present on merozoites, the addition of an antagonist such as atropine could also cause parasite death. The possibility also exists that AChR are present, but atropine does not affect viability of the parasite because it cannot bind to the receptor for mechanical reasons due to merozoite anatomy. At the highest concentration used in this study, 10 mM, atropine sulfate caused a statistically significant decrease in plaque numbers. However, complete absence of plaque growth, as seen in merozoites exposed to concentrations of pyrantel tartrate greater than 2.5 mM[1],

did not occur. The decrease in plaque numbers does not appear to be a result of exposure to the sulfate portion of the molecule, for numbers similar to that of the DMEM control were seen with exposure to sodium sulfate. It cannot be ruled out that the decrease in plaque numbers seen with atropine exposure may be a result of the drug working at the ACh receptor; however, because of the lack of response to the ACh molecule itself, it is most strongly suggested that atropine sulfate is effective against *S. neurona* merozoites through an action separate from that of the AchR and that this action needs further exploration.

If pyrantel tartrate did work at the AChR in *S. neurona* merozoites, theoretically, a AChR antagonist like atropine could bind to the receptor and prevent the cascade of events leading to merozoite death that follows exposure to pyrantel tartrate (or acetylcholine). However, in order to test this theory, atropine molecule itself must not affect parasite viability. In this study, atropine sulfate did not decrease viability at any concentration with the exception of 10 mM. Exposure of merozoites to atropine sulfate prior to pyrantel tartrate application did not cause a reversal of the lethal effects of the drug on merozoites with any atropine concentration. The fact that this reversal did not occur is further evidence that ACh receptors are not present on *S. neurona* merozoites and that pyrantel tartrate works at a novel drug target in this parasite. The possibility does exist that merozoites were not exposed to atropine for a long enough period of time for the drug to bind. Additional experiments using time of atropine exposure as a variable should be completed before the possibility of using an antagonist to block pyrantel activity is dismissed.

The acetylcholine receptor is a 250kDa pentameric complex of four transmembrane subunits in a stoichiometery of $\alpha_2\beta\gamma\delta$[15]. The ACh binding site is primarily in the α subunit. Monoclonal antibodies against this protein did not produce a 49 kDa band on Western blot prepared with *S. neurona* merozoite lysate while a band was seen at this molecular weight with the BC3H1 lysate control. The positive blot control indicated the blot was prepared adequately. Because the protein of the AChR α subunit was not detected with Western blot, it is likely that this protein, which acts as the primary binding site for ACh, is not present in *S. neurona* merozoites. It is possible that the monoclonal antibody used in this study, because it was prepared from the mammalian AChR, is not compatible with the protozoal AChR or that the protozoal receptor differs from the mammalian receptor to a degree that the antibody cannot sufficiently bind. Even so, there is little evidence from this study that supports *S. neurona* merozoites having acethycholine receptors, and the absence of a detectible AChR α subunit protein in merozoites on Western blot adds to the theory that the receptor is simply not present in this stage of the parasite.

Further biochemical and molecular testing is necessary to rule out the presence of ACh receptors on *S. neurona* merozoites, as well as to determine the drug target of pyrantel tartrate in this parasite. This initial study has found no evidence to support the theory that *S. neurona* merozoites possess ACh receptors.

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	ACh chloride	Simultaneous 95% Confidence Interval	Atropine sulfate	Simultaneous 95% Confidence Interval
Week 1	Difference between means		Difference between means	
DMEM	-	-	-	-
0.001 mM	-9.50	-23.62, 4.62	-8.25	-26.36, 11.36
0.01 mM	-14.25	-28.37, -0.13*	-10.00	-28.36, 8.36
0.1 mM	-1.00	-15.12, 13,13	-7.00	-25.36, 11.36
1 mM	-1.75	-15.87, 12.37	-12.00	-30.36, 6.36
10 mM	-15.50	-29.62, -1.38*	-54.25	-72.61, -35.89*
Week 2				
DMEM	-		-	
0.001 mM	-34.50	-76.94, 7.94	-104.33	-139.98, -68.69*
0.01 mM	-23.75	-66.19, 18.69	-10.67	-46.31, 24.98
0.1 mM	-10.75	-53.19, 31.69	-4.67	-40.31, 30.98
1 mM	-19.00	-61.44, 23.44	-3.67	-39.31, 31.98
10 mM	-18.75	-61.19, 23.69	-7.67	-43.31, 27.98

Table 5.1. Results of Dunnett's t-tests comparing each dose group to the DMEM control at alpha = 0.05. All results are based on n = 4 plates per group, except for atropine sulfate week 2, where n = 3 plates per group. * Indicates a significant difference from the control at $p \leq 0.05$.

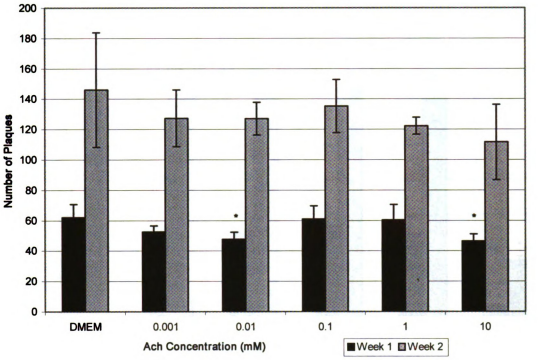

Figure 5.1. Acetylcholine plaque assay results. Plaque numbers (mean of four wells ± standard deviation) one and two weeks post-inoculation. Concentrations of 10^{-6}M and 10^{-3}M were significantly lower than the DMEM control after one week of growth; however, no statistical difference was seen between the media control and treatment groups at all concentrations after two weeks of growth.

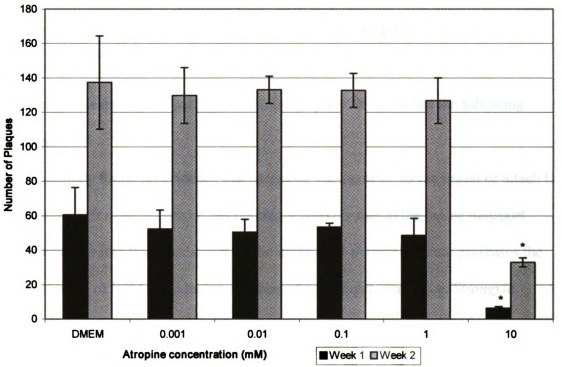

Figure 5.2. Atropine plaque assay results. Plaque numbers (mean of four wells ± standard deviation) one and two weeks post-inoculation. No statistical difference between media control and treatment groups at all concentrations after two weeks of growth.

CHAPTER 6

MAJOR FINDINGS AND CONCLUSIONS

From the experiments performed in this study we have reached the following conclusions:

1. This study found no evidence that *Strongyle* L_1, L_2, or L_3 larvae ingest or attach to *Sarcocystis neurona* sporocysts making it unlikely that a) the larvae transport sporocysts into the bloodstream of the horse or b) pyrantel tartrate decreases the incidence of EPM by reducing numbers of an indirect carrier (i.e. *Strongyle* larvae).
2. Pyrantel tartrate is 100% lethal to *S. neurona* merozoites at concentrations greater than 2.5 mM. This indicates that the drug may work on the parasite directly to prevent EPM; however, the merozoite stage of the parasite is found in the CNS of horses, and pyrantel is not absorbed from the GI tract, making exposure of this stage of the parasite to the drug highly unlikely *in vivo*.
3. Pyrantel tartrate is lethal to *S. neurona* sporozoites at the concentration of 5 mM. This stage of the drug is found in the GI tract and is the stage most likely to be exposed to pyrantel; however, the concentration of 5 mM is much greater than the actual drug concentration found in Strongid®-C. The possibility remains that the drug is effective in preventing EPM because it is lethal to sporozoites. The next step in proving this theory is to test the drug in a horse model.
4. Pyrantel tartrate increases the mean survival time of gamma interferon knock-out mice when given before, during, and after oral *S. neurona* sporocyst dosing.

Additionally, oral dosing with the drug changes the infection pattern in these mice by decreasing the incidence of encephalitis. Again, the potential for disease prevention is present, but experiments using an equine model are necessary before any conclusions can be made about the efficacy of Strongid®-C in the prevention of EPM.

5. This study found no evidence that *S. neurona* merozoites possess acetylcholine receptors. Further testing is required to determine the drug target of pyrantel tartrate in *S. neurona*.