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ABSTRACT

*Sarcocystis neurona* is the etiologic agent of equine protozoal myeloencephalitis, a neurologic disease of horses. The present study was designed to test the hypothesis that pyrantel tartrate can kill *S. neurona* merozoites growing in equine dermal cell culture. *Sarcocystis neurona* merozoites were exposed to a range of concentrations of pyrantel tartrate or sodium tartrate ranging from 0.001 to 0.01 M. Merozoites were then placed onto equine dermal cell cultures and incubated for 2 weeks to check for viability. At 1 and 2 weeks after inoculation, plaque counts were compared between treatments and, between treatments and controls. Merozoites exposed to concentrations of pyrantel tartrate higher than 0.0025 M (8.91 × 10⁻⁴ g/ml) did not produce plaques in equine dermal cells, whereas 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 equine gut.

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INTRODUCTION

*Sarcocystis neurona* is the etiologic agent of equine protozoal myeloencephalitis (EPM), a neurologic disease of horses.¹ Infection of the central nervous system (CNS) of equids with this protozoan parasite can produce a variety of clinical signs including weakness, ataxia, and lameness.¹ The life cycle 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 recently been found to be an 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 Sexual replication of *S. neurona* occurs in the intestine of the definitive host.

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host, the opossum (*Didelphis virginiana*). 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. Horses and ponies become infected when they ingest sporocysts from opossum feces, but *S. neurona* does not form tissue cysts in equids. It is likely that sporozoites are excysted from sporocysts in the digestive tract of horses, 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.

Equine protozoal myeloencephalitis is considered the most important protozoal disease of horses in the Americas. Exposure to *S. neurona* is high, with a seroprevalence in horses in some areas exceeding 50%. This high exposure rate, in addition to the serious consequences 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 in Strongid® C (Pfizer, New York, 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 the present study was to determine whether pyrantel tartrate has activity against *S. neurona* merozoites. The merozoite stage of the parasite used in this study replicates in the CNS of horses and is not likely to be exposed to pyrantel tartrate, which has low absorption and remains primarily within the digestive tract. However, it is believed that results obtained from merozoite studies may be useful to model how the drug will work against the sporozoite stage of *S. neurona* in the digestive tract. Positive results would lead to consideration of further studies investigating the possibility of using pyrantel tartrate as a preventative for EPM.

**MATERIALS AND METHODS**

**Preparation of Merozoites**

*Sarcocystis neurona* merozoites were grown and maintained on low-passage (1–19) equine dermal cells (American Type Culture Collection CCL57, strain NBL-6) with DMEM (Dulbecco’s modified Eagle’s medium supplemented with 6% heat-inactivated fetal bovine serum, penicillin [100 U/ml], amikacin [100 µg/ml], and amphotericin B [1.25 µg/ml]). Medium containing asexually replicating stages of the parasite was removed from four heavily infected flasks (approximately 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 \( \times \) 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 hemacytometer. The mean and standard deviation of these counts were determined. The merozoite count of the stock solution was 2.77 ± 0.13 \( \times \) 10^6/ml. A 200-ml 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 \( \times \) 10^5.
Cell Culture Preparation

Equine dermal cells harvested from passage 19 were grown to confluency as determined by visual examination using an inverted microscope in eight 6-well cell culture plates.

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 for EPM, and sodium tartrate (Sigma, Inc.) was used as a salt control. A 0.1-M solution (3.564 × 10⁻² g/ml) of pyrantel tartrate 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 0.01, 0.0075, 0.005, 0.0025, and 0.001 M. A 0.1-M solution (2.30 × 10⁻² g/ml) of sodium tartrate 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. One ml of DMEM alone (pH 7.88) was added back to two additional tubes as a negative control.

Experimental Design

Tubes prepared as above were incubated at 37°C in 5% CO₂ for 24 hours, then centrifuged for 10 minutes at 209 × g. The supernatant was carefully removed to prevent pellet disruption, 1.0 ml of DMEM was added back to each tube as a washing step, and the 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 among four wells (1.0 ml per well) of six-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% CO₂ for 24 hours, after which all media were aspirated from the wells. Two milliliters of fresh DMEM was added back to each well and aspirated as a washing step. Three milliliters of DMEM was then added to maintain the cultures. DMEM in all wells was changed weekly for the duration of the experiment.

Comparisons of Plaque Numbers

After 1 week, equine dermal cell monolayers were observed for plaques, which were counted with an inverted microscope (4× 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 in each pyrantel tartrate and sodium tartrate dilution (four wells per dilution) were compared with numbers in the DMEM control (four wells) using the Wilcoxon rank-sum test (exact test, two-sided). In addition, plaque numbers for each pyrantel tartrate dilution were compared with those in sodium tartrate dilutions. For all comparisons, significance was declared when *P* < 0.05. Plates were returned to the incubator after the first count, and plaques from each drug dilution were again counted 1 week later and compared as before.

DNA Extraction and Polymerase Chain Reaction

Cell culture supernatants and cell monolayers were both tested for the presence of *S. neurona* DNA using polymerase chain reaction (PCR). Medium was removed from all four wells of each drug dilution after 2 weeks and combined in 15-ml conical tubes (one tube per dilution). This media was centrifuged for 15 minutes at 1877 × 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 milliliter of alkaline chelating solution (ACS) was added back to the monolayer of cells in each well. The plate was gently rocked to allow the monolayers to detach from the wells and become suspended in the ACS. The suspension from all four wells of each dilution was similarly removed and combined. The suspensions were then centrifuged for 15 minutes at 1877 \times g, the supernatants removed, and 0.5 ml of PBS added back to the pellets. The suspension from each tube was 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 refrozen with dry ice three times prior to extraction. DNA was extracted from each sample with a Qiagen DNeasy™ Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instructions.

Polymerase chain reaction, 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. Briefly, PCR was performed under standard reaction conditions (50 nM KCl; 10 mM tris-HCl; pH 9.0 at 25°C, 0.1% Triton X-100; 1.5 mM MgCl2; 0.2 mM each deoxynucleotide triphosphate) with a GeneAmp® PCR System 9600 (PerkinElmer, Wellesley, MA) thermocycler with the following conditions: 72°C, 3 minutes; 35 cycles of (94°C, 30 seconds; 37°C, 1 minute; 72°C, 45 seconds); 72°C, 6 minutes; 4°C, hold. Polymerase chain reaction products were visualized and compared on a 1.8% agarose gel with ethidium bromide staining. These samples were tested on two separate occasions.

### RESULTS

#### Plaque Numbers

All pyrantel tartrate dilutions produced lower plaque numbers when compared with the DMEM control after 1 and 2 weeks (*P* < .03) (Table 1; Figures 1 and 2). Merozoites exposed to pyrantel tartrate concentrations of 0.005, 0.0075, and 0.01 M produced no plaques after 1 and 2 weeks. Merozoites exposed to 0.0025 M pyrantel tartrate produced no plaques after 1 week and only a small number of plaques after 2 weeks. Merozoites exposed to pyrantel tartrate at 0.001 M produced plaques, although significantly fewer than that of the control medium (*P* < .03).

All sodium tartrate dilutions and the DMEM control had similar numbers of plaques (*P* > .05) after 1 and 2 weeks (Table 1; Figures 1 and 2). All pyrantel tartrate dilutions produced low-
er numbers of plaques than sodium tartrate dilutions of similar molarities ($P \leq .03$), with the exception of 0.001 M after 2 weeks ($P = .20$) (Figures 1 and 2). The number of plaques for the DMEM control group was not significantly different ($P > .05$) after 1 and 2 weeks.

**Polymerase Chain Reaction**

A strong *Sarcocystis* PCR product was only detected in medium removed from the lowest concentration of pyrantel tartrate (0.001 M). A weak *Sarcocystis* PCR product was detected in medium removed from pyrantel tartrate concentrations of 0.0025 M and higher. In contrast, a strong *Sarcocystis* PCR product was detected in the medium of all sodium tartrate treatment groups and DMEM controls (Figure 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).

**Drug Efficacy Control**

The number of viable third-stage strongyle larvae decreased with exposure to all concentrations of pyrantel tartrate compared with numbers in the DMEM control ($P < .0001$) (Table 2).

**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 schizonts) were exposed to various concentrations of pyrantel tartrate for 24 hours to determine if the drug had activity against this parasite. Pyrantel tartrate appeared to be completely lethal to S. neurona merozoites at concentrations greater than 0.0025 M, as there was no plaque growth when merozoites were added to cell cultures after exposure to these concentrations. At 0.0025 M, no growth occurred after 1 week in culture, and limited growth was seen after 2 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 0.0025 M, 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, 2001). The weak PCR product detected in medium and cells removed from wells with zero or few plaques may be due to small amounts of residual DNA from nonviable organisms trapped in the equine dermal cell monolayer. The fact that PCR products from wells with large plaque numbers

Figure 1. Plaque numbers (mean of four wells + standard deviation) 1 week after culture inoculation. *Significant difference between sodium tartrate and pyrantel tartrate treatment (P < .03).

Figure 2. Plaque numbers (mean of four wells + standard deviation) 2 weeks after culture inoculation. *Significant difference between sodium tartrate and pyrantel tartrate treatment (P < .03).
showed a greater amplification of DNA indicated that 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, it could not determined 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 those of pyrantel tartrate showed growth similar to that of the DMEM control in culture, and PCR detected a strong *Sarcocystis* PCR product in all concentrations of this compound. These results lead to the conclusion that merozoite death observed with pyrantel tartrate was due to the presence of the tartrate compound and not to the presence of DMEM or the tartrate salt alone. Additionally, the decrease in viability of third-stage strongyle larvae after exposure to concentrations of pyrantel tartrate at and below that which inactivated merozoites shows that the drug was active at all concentrations used in this study.

Comparison of pyrantel tartrate and sodium tartrate dilutions indicates, with the exception of 0.001 M at 2 weeks, the number of plaques produced by the sodium tartrate dilutions was significantly greater than that for the pyrantel tartrate dilutions. This dilution (0.001 M) of pyrantel tartrate produced significantly fewer plaques than the DMEM control; however, the number of plaques produced was higher than numbers produced at higher concentrations of the compound. This indicates that at the dose of 0.001 M, 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 demonstrated. The dosage rate for pyrantel tartrate given daily to equids in Strongid®C is 1.2 mg/lb of body weight, making the daily dose 1.2 g for a 1000-lb horse. This dosage provides a concentration of pyrantel tartrate much greater than that determined to kill *S. neurona* merozoites in this study on a gram-for-gram basis. This provides 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 horses may vary, making it difficult to compare a gram per volume dose to the dosage tested in vitro in this study. Pyrantel tartrate was found to be ineffective against *S. neurona* infection in γ-interferon gene knockout mice; however, the digestive tracts and gastrointestinal transit times of mice and equine vary greatly. Additional in vivo studies will be needed to determine whether pyrantel tartrate protect horses from *S. neurona* infection.
Pyrantel tartrate is an anthelmintic compound that possesses nicotine-like properties, acting similar to acetylcholine (ACh), and inhibits acetylcholinesterase. These properties make the drug a depolarizing neuromuscular-blocking agent in susceptible parasites. Exposure to pyrantel tartrate leads to paralysis of the susceptible organism. The presence of ACh has been detected in bacteria and primitive organisms such as blue-green algae, yeast, fungi, tubellaria, protozoa (Trypanosoma rhodesiense, Paramecium), nematodes, and sponges. In studied organisms, ACh occurs in both neuronal and nonneuronal tissues. Nonneuronal ACh appears to be involved in the regulation of basic cell functions, such as proliferation, differentiation, cell–cell contact, immune functions, secretion, and absorption. It has been speculated that ACh may be involved in the rapidly moving protozoa-like trypanosomes but not in sluggish ameboid movement. 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, which remains primarily in the digestive tract. However, because the merozoite stage has some similarities to the sporozoite stage found in the gut of horses and is readily available for in vitro studies, the present study was designed to examine 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.

## CONCLUSION

Pyrantel tartrate at concentrations greater

| TABLE 2. Viable and Nonviable Strongyle Larvae after Exposure to Pyrantel Tartrate |
|---------------------------------|---------------------------------|---------------------------------|------------------|------------------|------------------|
| **Concentration**              | **Viable Larvae**               | **Nonviable Larvae**            | **%**             | **P value**      |
| DMEM                           | **Before Pyrantel Exposure**    | **After Pyrantel Exposure**     | **After Pyrantel Exposure** |                  |
| 0.0000001 M                    | 8                               | 7                               | 1                | 12.5             | NA               |
| 0.000001 M                     | 18                              | 1                               | 17               | 94.4             | <.0001           |
| 0.0001 M                       | 13                              | 0                               | 13               | 100              | <.0001           |
| 0.001 M                        | 16                              | 0                               | 16               | 100              | <.0001           |
| 0.01 M                         | 27                              | 1                               | 26               | 96.3             | <.0001           |
| 0.1 M                          | 14                              | 0                               | 14               | 100              | <.0001           |

*Number of nonviable larvae in pyrantel tartrate dilutions versus number in DMEM control (Fisher’s exact test).
DMEM = Dulbecco’s modified Eagle’s medium supplemented with 6% heat-inactivated fetal bovine serum, penicillin, amikacin, and amphotericin B; NA = not applicable.
than 0.0025 M (8.91 × 10^-4 g/ml) kills both free *S. neurona* merozoites and stages of the parasite asexually replicating within equine dermal cells. These results have prompted further investigations into the possibility of commercial application of pyrantel tartrate as a preventative for EPM in horses.

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**REFERENCES**


