In vitro efficacy of anti-protozoal compounds as a novel treatment of *Tritrichomonas foetus*

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Abstract

Bovine trichomoniasis is a sexually transmitted disease caused by *Tritrichomonas foetus*, an obligate parasite of the reproductive tract of cows and the surfaces of the penis and prepuce of bulls. With the ban of efficacious drugs for treatment of *T. foetus* such as the nitromidazoles in the United States, veterinarians are currently left without legal means of treatment. Oxfendazole, oxibendazole, and ponazuril are anti-protozoal drugs that can be legally used in food animals. *In vitro* experiments were performed using pure cultures of *T. foetus* trophozoites in Diamond's medium with the addition of the individual drugs in replicate. Cell cultures were evaluated at predetermined time points using Neubauer hemocytometers to determine the number of viable trophozoites. Cultures were examined for reversibility to the motile trophozoite form or continued replication of organisms following the administration of the drug. In our final study, complete kill of the organism was achieved and all replicates remained negative for reemergence of trophozoites following five days of culture.

Keywords: *Tritrichomonas foetus*, bull, benzimidazole, ponazuril, treatment

Introduction

Tritrichomonas foetus, an extracellular flagellated protozoan, is the organism responsible for the sexually transmitted disease commonly known as bovine trichomoniasis. This organism is an obligate parasite of the female reproductive tract and surface of the male's prepuce and penis in cattle.[1] *Tritrichomonas foetus* is a pleomorphic organism that can exist in both the trophozoite and pseudocyst form both of which can be transmitted during coitus.[1-4] The bull, an asymptomatic carrier of the *T. foetus* organism, transmits the protozoan to the female during coitus with reports of a single mating with an infected bull inducing an infection in 95% of susceptible nulliparous cows.[5] Infected cows may experience vaginitis, pyometra, embryonic death, and abortion.[6]

The impact of decreased calf crops and prolonged calving seasons that occur as a sequela to the clinical infection in the cow can be quite substantial as infection rates amongst herds have been reported from 5-50% with variability based upon geographical location.[7-10] Speer and White speculated that a 5% calf loss due to trichomoniasis in US beef cattle herds would translate into 1,685,250 calves lost based on 1989 calf production as estimated by the USDA.[11] This equated to a $650 million annual loss to the industry. The USDA has estimated the United States calf crop to be approximately 35.1 million in 2016.[12] Assuming a five percent loss of calves due to *T. foetus* infection at current market prices, the loss to the industry would approach 1.5 billion dollars.

While most cows mount a short term immune response and eventually clear the infection for the year, the bull fails to establish an immune response to the infection allowing for the establishment of the chronic carrier status.[13] This fact combined with the severe economic impact due to loss of calves along with the current lack of a legal effective treatment all contribute to the decision that bulls found positive for the disease are culled from the herd as part of disease management. This results in additional losses to the producer in the form of monetary expenditures of replacing the bull, and in some cases the loss of valuable livestock genetics. With the increasing value of cattle, the opportunity to treat bulls infected with *T. foetus* has once again become a priority for cattle producers as opposed to culling and replacing infected bulls.

Several treatments for trichomoniasis were used successfully in the past, including: Bovoflavin Salve® (Farbwerke Hoechst AG, Frankfurt, Germany), ipronidazole, and metronidazole.[14] Bovoflavin

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Salve® contained trypaflavine at a concentration of 0.5% and was applied to the penis and prepuce of infected bulls. When the salve was applied to 57 bulls, 55 bulls proved to be cured by examination two to four weeks following treatment.[14] Bartlett treated eight infected bulls with Bovoflavin Salve® and found that seven were cured by a single series of treatment.[15] Nitromidazoles were also reported to have similar successes when applied topically.[16] Unfortunately, Bovoflavin Salve® is no longer manufactured and nitromidazoles became illegal for use in food animals.[17]

The objective of this project was to investigate *in vitro* the use of novel therapeutic drugs for the treatment of *T. foetus*. Extrapolating from the previous treatment successes of the use of topical treatments such as Bovoflavin Salve® and nitromidazoles, novel therapeutic dosage formulations were tested *in vitro* for their ability to kill or reduce the number of *T. foetus* organisms in culture. Various formulations were tested using two types of antiprotozoals, ponazuril (PO) and benzimidazoles including oxibendazole (OX) and oxfendazole (OXF). If found to be successful these antiprotozoal drugs would be utilized to form a novel chemotherapeutic that could be applied to the prepuce and penis of infected bulls in similar fashion to the successful Bovoflavin Salve®.

Benzimidazoles were chosen as a potential treatment for *T. foetus* as they specifically affect the colchicine binding-site of β-tubulin monomers inhibiting microtubule assembly and disassembly.[18,19] Microtubules play a vital role in the cytoskeleton of the trichomonad. The pelta, flagella, spindle and basal bodies as well as the axostyle, the organelle responsible for cell division, are all formed from these structures.[20-22] Moreover, benzimidazoles can also act as a lipid-soluble proton ionophore, inducing direct and indirect biochemical changes including inhibition of glucose uptake, glycogen depletion, inhibition of the fumarate reductase system and uncoupling of electron transport-associated phosphorylation.[23] The exact mechanism of action the triazine agents such as PO is currently unknown. Totrazuril sulfone, more commonly known as PO, has been found to have clinical application in the treatment of *Neospora caninum* in cattle and may have clinical application for treatment of other protozoal disease in cattle including *T. foetus*.[24]

Materials and methods

Experimental design

Two i*n vitro* experiments were performed using pure cultures of *T. foetus* trophozoites. In the initial study OX (Anthelcide EQ® Zoetis, Florham Park, NJ) or PO (Marquis® Merial, Duluth, GA) paste formulations were utilized. In the second study OXF powder (U.S. Pharmacopeial Convention Rockville, MD) was utilized. Samples were evaluated at predetermined time points using Neubauer hemocytometers to determine the number of viable, motile organisms. Live trophozoites were determined as those protozoa exhibiting pear shaped bodies, externalized flagella and displaying motion. Non-motile trophozoites were characterized by the presence of the pear shaped bodies with externalized flagella but lack of motion and were not counted. Cultures were tested for reversibility to the motile trophozoite form or replication of organisms.

Parasites

The CDTf3 strain of *T. foetus*, a pure culture cultivated at Auburn University, was used in this study. The parasites were cultivated in trypticase–yeast extract-maltose Diamond's medium (DM),[25] supplemented with ten percent heat-inactivated fetal bovine serum (Atlanta Biologics, Norcross, GA) at 37˚C for 24-48 hours until appropriate numbers of trichomonads were present to perform the required number of replications for the study. Following culture, *T. foetus* organisms were washed in sterile phosphate buffered saline (PBS;VWR, Radnor, PA) centrifuged at 4000g for ten minutes at room temperature and re-suspended in 1 mL of PBS and then inoculated into 15 mL sterile conical tubes (BD Falcon, Franklin Lakes, NJ) or tissue culture wells (Sarstedt, Inc., Newton, NC) containing DM.

Statistics

Data were analyzed using statistical software package STATA SE v.14.2 (StataCorp, College Station, TX). Significance of treatment group, time, and their interaction were assessed by a mixed

effects model with treatment group as a fixed effect and replicate as a random effect within time. Differences between groups and times were assessed by comparisons of least squares means with Bonferroni adjustment for multiple comparisons. Statistical significance was set a $P<0.05$.

Drug susceptibility and reversibility experiment I

Trophozoites (3.75×10^4 cells/mL) washed in PBS were inoculated into DM for a total of 10mL of DM in each tube. At time 0, each of the following treatments were added to culture tubes: 1) control: 0.5mL DM; 2) 75 mg PO (0.5mL); 3) 50 mg OX (0.5mL). Five replicates were completed for each treatment. Following incubation at 37˚C, samples were taken every two hours for a total of 12 hours, the tubes were vortexed prior to removal of a 20μL sample. From these samples, the surviving organisms were counted utilizing disposable Neubauer hemocytometers (C-Chip®, NanoEnTek Inc., Pleasanton, CA). To evaluate if the antiprotozoal induced the formation of the pseudocyst stage or non-motile trophozoites that could be reversed and the organism would return to a motile trophozoite, at 12 hours following treatment each formulation was centrifuged at 4000g for ten minutes at room temperature. The supernatant fluid was removed and the pellet was re-suspended in 10mL of fresh DM without drug and evaluated microscopically daily for five days for the presence of *T. foetus* organisms in culture.

Drug susceptibility and reversibility experiment II

Trophozoites (3.75×10^4 cells/mL) were cultured as described above and added to tissue culture wells for a total of 3mL of DM in each well. At time zero, each of the following treatments was added to tissue culture wells: 1) control 4mL DM; 2) 150 mg OX (1mL) for a total of 4mL; 3) 450mg PO (1mL) for a total of 4 mL. Each treatment was applied to tissue culture wells in duplicate. Following growth at 37˚C on a rocker plate (Hoefer Inc., Holliston, MA), samples were collected every 4 hours for a total of 24 hours. A 20μL sample was removed from each well, and the surviving organisms counted utilizing disposable Neubauer hemocytometers. Cultures were microscopically examined every four hours for a total of 24 hours for the presence of *T. foetus* organisms in culture by counting organisms as described in Experiment I. At 24 hours after treatment and following counting of organisms, each formulation was removed from the tissue culture well and centrifuged at 4000g for ten minutes at room temperature. The supernatant fluid was removed and the pellet was re-suspended in 4mL of DM and placed once again in a tissue culture well before being re-incubated at 37˚C. This process was repeated every 24 hours for five passages (120 hours) to allow for re-emergence of any organisms.

Results

In Experiment I, PO and OX significantly inhibited parasite growth at concentrations of 75 mg mL^{-1} and 50 mg mL^{-1}, respectively. The variables of treatment group, time, and their interaction were statistically significant ($p<0.0001$). Counts for the control treatment were significantly greater than for PO and OX treatment groups ($p<0.0001$) but counts for PO and OX groups did not significantly differ ($p=0.99$). However, following re-culture at 12 hours and examination at 24 hours, reestablishment of motile organisms was observed. (Figure).

In Experiment II the concentration of benzimidazole was increased with the use of OXF solution at 150 mg mL^{-1}. Oxfendazole had substantial inhibitory effects on trichomonad growth in the culture following an incubation period of 4 hours and non-motile trophozoites were maintained for 24 hours and reversibility of organism to motile trophozoites was not demonstrated in continued cultures. Once again the variables of treatment group, time, and their interaction were statistically significant ($p<0.0001$). Cultures containing PO could not be evaluated as there was limited visibility when viewed microscopically and therefore removed from the study.

Discussion

In the present study, the *in vitro* vulnerability of bovine *T. foetus* to PO, OX, and OXF was evaluated utilizing susceptibility and subsequent reversibility tests. The efficacy of each anti-protozoal medication was evaluated using a susceptibility technique. The time of sampling varied between the two

studies due to the fact that no significant difference was noted at evaluation between two hours and four hours in Experiment I and between four and eight hours in Experiment II. In Experiment I as hypothesized by the authors, OX and PO in the form of approved drug formulations for horses of Anthelcide EQ® and Marquis® respectively demonstrated an antiprotozoal effect on *T. foetus*, by severely inhibiting the growth of organisms in the culture. Susceptibility tests determining growth inhibition and reversibility tests demonstrated statistically similar results between OX and PO with OX causing a sharper decline in organisms as compared to PO.

In an attempt to induce a permanent reduction in organisms in Experiment II, OXF was substituted for OX to increase concentration of drug per culture without dilution in the oil-based suspension formulation. When the OXF solution was added to culture, no motile trophozoites were detected four hours after inoculation. Following continued incubation, the culture continued to remain negative for motile trophozoites. As well, tissue culture wells and a rocker plate were utilized in this experiment to ensure that the drug continued to mix within the media and did not settle out of the suspension.

In conclusion, the described experiments demonstrated that OXF solution inhibited the growth of the bovine strain CDTf3 of *T. foetus in vitro* and may offer a new treatment option. Limitations of this study include not using video microscopy or transmission electron microscopy to further elucidate the effects of our novel chemotherapeutic on the morphology and ultrastructure of the *T. foetus* organism.

Acknowledgement

Conflict of interest

The authors have no conflicts of interest to report.

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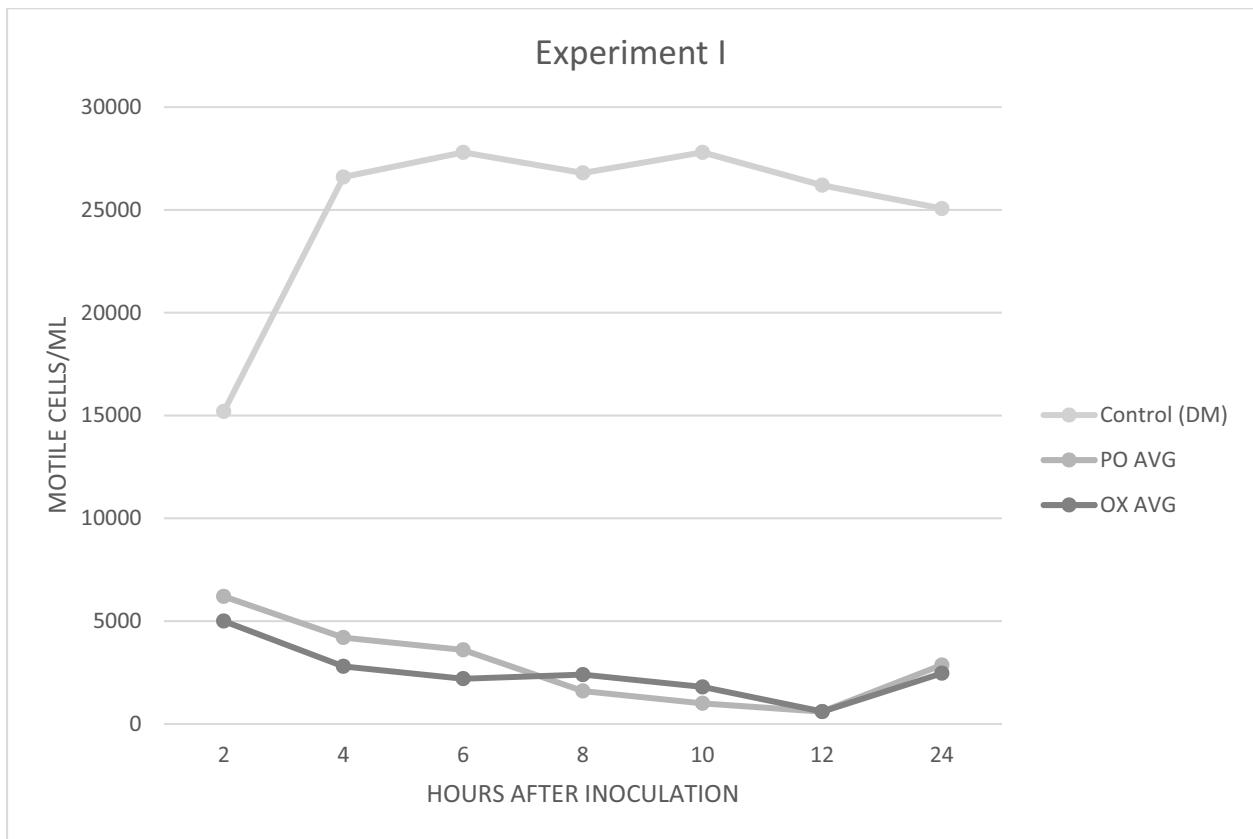
Figure. Averages for each culture were obtained by averaging the 5 replicates at the individual time points. Both PO and OX alone demonstrated a 98% reduction in motile organisms after two hours of culture. However, following re-culture at 12 hours and examination at 24 hours, reestablishment of motile organisms was observed.