Plasmodium falciparum Response to Oscillating Weak Magnetic Fields

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Executive Summary

Malaria is a disease that is endemic through the tropic and developing world. This parasitic infection kills individuals by consuming the hemoglobin in the blood. In this process, the parasite produces a paramagnetic waste hemozoin. Hemozoin then remains inside the erythrocytes until it is lysed by the parasite, 12-24 hours later. Hemozoin's paramagnetic properties make it of keen interest of study for detection and treatment possibilities. This report seeks to examine hemozoin's role in parasite growth. In particular, how does applied a weak alternating magnetic field to a cell culture containing these paramagnetic crystals effect its growth? The malaria parasite, *Plasmodium falciparum* exhibits a life cycle which is quite complex. In each life cycle the creation and placement (intra versus extracellular) of the hemozoin is different. It was found by researches Henry Lai *et al* that the application of a weak, slow, alternating magnetic field was able to vibrate the hemozoin within the parasite to kill it. Similar work done by colleague Dr. Robert Deissler showed that in fact the effect of a similar alternating magnetic field produced more parasites in a particular growth stage especially at the 24 hour mark. In this experiment, the growth under alternating magnetic fields is studied. Solenoids of various turns in series with a function generator were placed around an infected blood sample and grown in an incubator for 56 hours. At 24 hour intervals, subsamples were taken. Microscopy is used to determine the parasitic composition of the sample. This measurement indicates how many cells are dead, alive, and in what parasitic life stage they were found in. It was found that the growth of the parasites was significantly inhibited by the magnetic treatment. No matter the background growth, the treatment groups always grew far worse than the control groups. The next step is to understand what mechanism causes the parasites to die in the presence of a magnetic field.

Introduction

Today, malaria is one of the largest challenges to public health and global development. This parasitic infection is deeply woven into the fabric of the underdeveloped i endemic societies. It is both a cause and effect of poverty. Those who are poor or live in county of limited infrastructure may be unable to sleep under bed nets, spray DDT or drain standing water: the most effective ways of preventing malaria. Furthermore, an individual stricken with malaria is unable to earn an income and a state with a high infection rate become even less economically productive. According to the World Health Organization's 2009 World Malaria Report, there are 108 malaria endemic countries [1]. Malaria is a major force in reinforcing the cycle of poverty. To reverse the effects of malaria, new and better ways of identifying and treating the disease need to be derived.

In general, the three routes to take in addressing the world malaria problem are: prevention, detection, and treatment. Investigating the properties of the byproduct of malaria digestion of red blood cells, called hemozoin, seeks to improve upon detection and treatment. Currently, the best way to detect a malaria infection in the field is by microscopy. This involves the staining of blood using Gimesa and visual investigation by microscope. The problem with this technique is that staining and identifying the parasite requires a skilled technician, which is not always available. Significant levels of error have been shown with microscopic diagnosis of malaria with false positive rates reaching as high as 36% and false negatives as high as 18% of the time [2]. Because of this high rate of error, there is a great need for diagnostic techniques that can be accurately operated and understood without any training.

In addition, in the past two decades, the spread of drug resistant malaria has increased. Currently, the most commonly used drug is Chloriquine. It is thought that this drug may work

by inhibiting the formation of hemozoin to allow the buildup of toxic free iron particles. . This chloroquine-resistant strain originated in South East Asia but has spread to Africa. Presently, the majority of deaths due to this strain are among African children.

A key piece in both of these efforts is in understanding the effect of hemozoin, the paramagnetic waste product of the malaria parasite. Because the iron in the hemozoin crystal is has a higher spin than the iron in hemoglobin, blood with and without hemozoin (or infected and uninfected blood) should demonstrate different electromagnetic properties. Similarly, because of the presence of such magnetic tissue, infected blood should exhibit some change when placed under a different electromagnetic environment.

Specifically, this project aims to show the effect of immersing a sample of infected blood in a weak alternating magnetic field. The hypothesis is that a malaria parasite in a changing magnetic environment will undergo a change in its growth pattern that will eventually lead to its death. The objective of this experiment is to perfect a mechanism that will most effectively kill the parasite, while furthering the understanding of the role of hemozoin. This will make better known the role of hemozoin in the life cycle of *Plasmodium falciparum*. Furthermore, if such a process can be made to effectively and consistently kill parasites, it may be engineered into an alternative treatment technique.

Biology Background

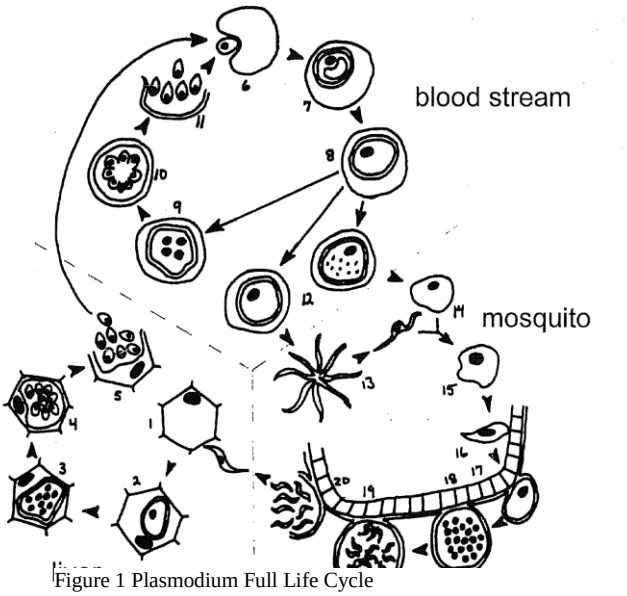


Figure 1 Plasmodium Full Life Cycle

Plasmodium faliparum uses a fairly complicated life cycle. The three major phases are the mosquito phase, the human liver phase, and the human blood stream phase. This project focuses on the blood stream phase. After infection via a bite from a female mosquito, the parasite infects the liver. Here exoeurythrocytic schizont, a type of asexual reproduction, releases merozites into the bloodstream. **Merozoites** enter the red blood cell and reach a stage where they simply grow and consume red blood cell cytoplasm; in this early trophic stage the trophozoites are commonly called "**ring form**". The later trophic stage where the parasite begins to consume a significant part of the cytoplasm is called **trophozoite**. In the next stage, the parasite undergoes multiple nuclear divisions without cytokinesis resulting in **schizonts**. When the parasites bud off from the original host cell, they again become merozoites and the cycle continues. The parasite has up to 60 seconds to invade another red blood cell host before it dies. In *Plasmodium falciparum*, the parasitemia (percentage infection) exhibits 48 hour periodicity.[3]

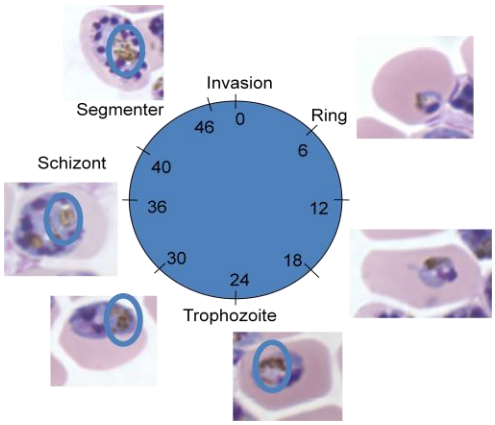
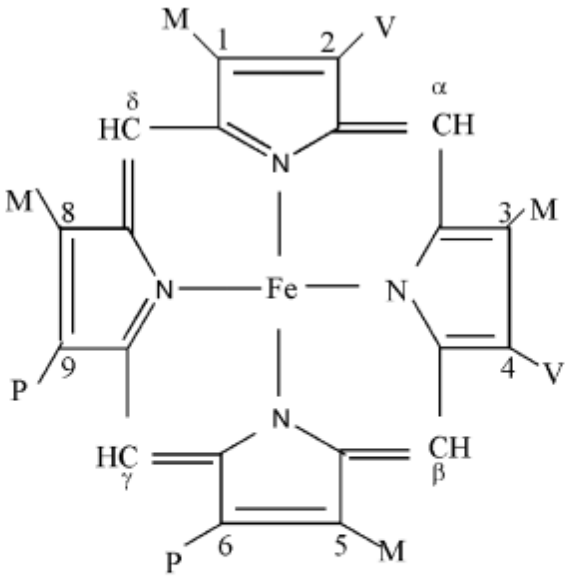


Figure 2Plasmodium falciparum Blood Life Cycle

There is a normal trend this life cycle takes with hieightened and subdued parasitima levels. If magnetic fields are applied, they would not be expected to affect all stages of the

parasitimaasite equally. Most importantly, when the parasite leaves the schizonts stage and reenters the merozoites stage, it dumps its hemozoin load into the extracellular fluid.

In the trophic stage, the malaria parasite consumes the innards of the red blood cell. Largely, this involves the digestion of hemoglobin, the iron-containing molecule responsible for binding with oxygen for oxygenation of the body. The globin part of the protein is processed into amino acids for protein synthesis or energy. The iron containing group, the heme(orferriprotoporphyrin IX) is not digested because the parasite does not have the enzyme hemeoxygenase. Free heme is very toxic to the parasite. In this 48 hour cycle, *Plasmodium falciparum* is able to consume between 50 and 80% of the cytocolic hemoglobin releasing of between 10-16mM of heme.[4] Heme is an Na^+/Ka^+-ATPase inhibitor and can cause a chain reaction of oxygenation of unsaturated fatty acids involving free radicals which can lead to membrane damage. [6] Heme can also cause covalent cross-linking and formation of proteins and its degradation of small peptides. Furthermore, because it is a lipophiolic molecule, it is able to intercalate in membranes and destabilize a cytoskeleton. Heme may even damage DNA through oxidative stress.[5] While the physiology of the human body has



Heme (Ferroprotoporphyrin)

M = —CH_3
V = —CH =CH_2
P = —CH_2— CH_2—COOH

the capacity to detoxify free heme in a concentration on the order of 20 μM, *P. falciparum* does not. Overall, free heme has the destructive power to keep *Plasmodium falciparum* from living.

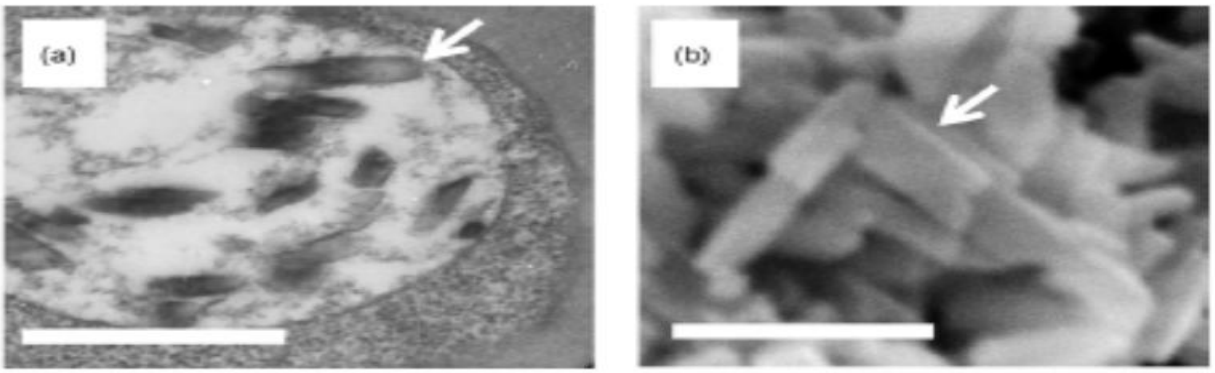


Figure 3Micrographs of hemozoin (indicated by white arrows) shown using transmission electron microscopy of a malaria parasite (A) and scanning electron micrograph of extracted crystals (B)

To survive, the malaria parasite stores the heme in an acidic digestive vacuole with of a pH of between 5.0 and 5.4 [9]. Here, the heme form dimers via an ionic bond between iron and oxygen. The dimer then polymerizes with the assistance of heme polymerase. Because of the close proximity of the oxidized iron, this crystal is paramagnetic. [6]

Literature Review

At University of Washington Department of Physics, the Lai group theorized that the increased movement of hemozoin in the culturing of plasmodium would inhibit growth. They applied an alternating magnetic field of 1.5 milliTesla at 5Hz over a duty cycle of 1 second on and 1.5 seconds off. The malaria cultures were grown in blood in an incubator at 37°C for 48 hours. They then counted the parasitemia with light microscopy technique. They found that the growth was reduced between 30% and 67%. They also tested the viability of the parasite by measuring hypoxanthine incorporation rate. This test also showed decreased viability after

exposure to the magnetic field treatment.

	parasitemia				3H-hypoxanthine incorporation			
	exp. 1	exp. 2	exp. 3	exp. 4	exp. 1	exp. 2	exp. 3	exp. 4
magnetic field sample	2.8-3.1%	4.0-4.4%	1.4-2.1%	1.5-2.1%	ND	12369	5451	7648
control 1a	4.8-5.6%	5.2-7.6%	2.4-2.4%	3.1-4.1%	ND	19231	6189	8634
control 2a	5.1-6.2%	4.9-6.7%	3.2-3.4%	4.8-6.2%	ND	22069	8054	12473
% of control 1b	57%	77%	58%	40%		64%	88%	88%
% of control 2b	51%	67%	43%	31%		56%	68%	61%

a Control 1 is located in the incubator with the Helmholtz coil and control 2 is located in a separate incubator.

b % of control is the magnetic field sample value divided by the control sample value. For the parasitemias, the average of three samples was used for the calculations.

Figure 4 Lai results; Effects on of an oscillating magnetic field on P. falciparum viability

They proposed that this may occur because the applied magnetic field prevents hemozoin from being formed and free heme is free in the body of the parasite. Conversely, they suggest that it is possible that the alternating magnetic field rotates the entire hemozoin crystal causing mechanical damage to the late stage parasites. [6]

Lee Moore and colleagues tested parasitic growth rate in a 0.140 T/mm (1.72 T applied) magnetic field. They found that the magnetophoretic mobility of *P. falciparum* infected cells was significant, but does not exceed mobility of deoxygenated cells. Of all the stages of parasites, schiozont cells were more mobile than other parasite life stages. This was attributed to the known difference in gravitational sedimentation rates. Most importantly, they concluded that the "fraction of hemoglobin converted to hemozoin, calculated from magnetophoretic mobility of live erythrocytes and the known magnetic susceptibilities of their constituents, agrees with the fractions measure by biochemical and crystallographic methods on fixed specimens".[7] This research finding is important because it shows that infected erythrocytes do actually show reaction to the presence of a magnetic field. This gives reason to expect that an alternating magnetic field will be able to vibrate the hemozoin within the infected cell.

Preliminary Work

However, preliminary work done in collaboration with colleague Dr. Robert Deissler was not in complete agreement with the Lai paper. Using a Helmholtz coil with 1.8mT magnetic field at the same frequency and duty cycle, he recreated the experiment described and found that there was not an immediate decrease in parasitemia but a shift from trophozoites and schizonts to rings. This suggests that the magnetic field encouraged the parasites to remain in the ring stage longer or killed of late stage parasites more. However, after 48 hours, there was an overall decrease in parasitemia.

Table 1Deissler Results**[8]**

Tube Name	% Reduction in Total Parasitemia	% Reduction in Rings	% Reduction in Trophozoites	% Reduction in Schizonts
3d7 24hr control	0±3.35	0±2.54	0±10.63	0±14.16
3d7 24hr Helmholtz	-18.23±3.95	-24.08±4.44	0.12±9.84	-4.8±4.44
3d7 48hr control	0±18.06	0±21.29	0±20.51	0±10.66
3d7 48hr Helmholtz	46.85±7.88	42.87±9.59	38.13±1.25	62.71±6.27

These results beg the question what is the effect of different levels of applied magnetic field on the life cycle of red blood cell stage plasmodium? Because the life cycle is cyclical, what changes can be observed if data are taken at shorter intervals? If the experiment is continued, what trends will be shown? Will the parasites continue to die and if so will their population be able to recover? What if the magnetic treatment is stopped? Will the infection return suddenly or will the treatment have a lasting effect?

Methods

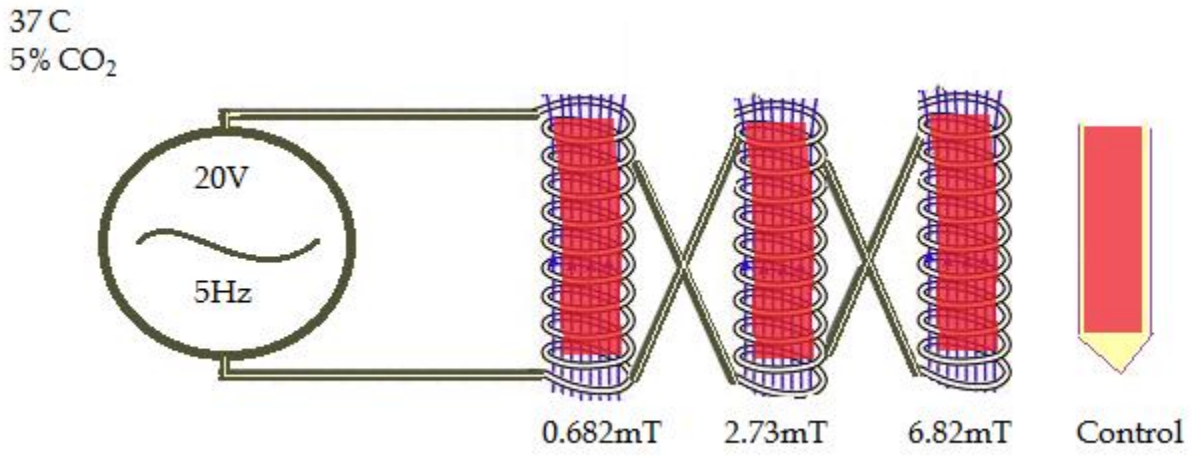


Figure 5 Apparatus

The methods for this experiment are similar to that of research previously performed by Lai et al. Parameters were chosen to best replicate their positive results. Instead of using a Helmholtz coil, solenoids were used to produce a more specially concentrated magnetic field. Three solenoids of 28 gauge wire were connected in series with different numbers of turns: {250, 1000, 2500}. This is done so that in one trial, several magnetic field strengths are tested above and below the magnetic field strength used by Lai and Deissler, 1.5mT. The former of this solenoid is a plastic titer-tube of diameter 1cm and length 3.5cm. A smaller tube holding the sample is placed inside the plastic former for the duration of the experimental trial. The solenoids will be connected with a function generator outputting variable amplitude up to 20 V peak to peak resulting in a current of 0.767mA RSM. This results in magnetic fields of 0.682mT, 2.73mT, and 6.82mT respectively.

Samples for treatment are selected from a culturing well containing the 3D7 strain of *Plasmodium falciparum* cultured using the Grimberg protocol[10] . These samples are approximately 3% hematocrit meaning that the volume of the sample is made up of 3% red blood

cells as opposed to extracellular fluid. To collect the sample, old media was aspirated off a culturing well. New warmed media was added, 4ml per well. Then 450μL was pipette out and added to the titer tube four times. It is important to note that this sample is asychronist. This means that it contains parasites in all blood-stage phases of their life cycle, principally: ring stages, trophozoites, and schizonts. This is important because the rings contain no hemozoin, trophs begin to produce hemozoin and schizonts have the most sequestered hemozoin. Therefore we expect that the treatment may affect each life stage differently. However, as long as the sample is asynchronous, we should expect to see some affect.

This apparatus will be placed inside an incubator at 37°C at 5% CO_2. Three treatment samples are placed in the three solenoids. A control sample will be placed inside the incubator away from the solenoids. A fan was blowing on the three coils to minimized Ohmic heating from the wires. The samples are fed with a complete media every 24 hours by aspirating off old media from the top f the settled sample and adding 400μL new warmed complete media. A 2μL sample is taken from the blood pellet to plate on glass slides at the 0^{th} hour, 24^{th} hour and the 48^{th} hour. Slide were labeled with strain, initials of experimenter, date, and slide name. The blood was smeared with another slide at a 45° angle to leave a thin film of blood. The slides were fixed in methanol for 20 seconds. They were then placed in 40 mL of 1XGeimsa buffer and 1.6 mL Geimsa stain for 30 minutes. This stain dyed the parasite morphology purple while leaving the red blood cells grey to pink. After the 30 minutes, the slides were rinsed using tepid water to reduce the number of stain artifacts seen during microscopy.

After the slides were made, the samples were maintained by gassing the sample tubes with CO_2 to reduce oxygen exposure and oxidative pressure. Then paraffin wax is thinly placed atop the samples to minimize debris. The paraffin also prevented loss of sample if the tubes

were inverted during transportation or if a coil tipped during the incubation. The tubes were then placed in the incubator in their respective tubes or control tube and left there to grow for 24 hours. After 48 hours was completed the samples were discarded in a biohazardous sharp waste.

Using light microscopy, up to 3000 red blood cells was counted per slide. The number of parasites in each life stage (ring, troph, or schizont) are counted. The number of dead parasite seen was also noted. Their growth rate is calculated and compared. The percentage increase relative to the 0^{th} hour was calculated for the 24 and the 48 marks.

The statistical error for parasitemia was determined using the following formula.

$$SE = \sqrt{\frac{p(1-p)}{n}}$$

Here *p* is the probability of a red blood cell being infected or the parasitemia. The variable *n* is the number of red blood cells counted in total to arrive at this parasitemia. The error of the percentage change was calculated using the derivative method and adding the error components in quadrature.

$$E_{\%\Delta} = \sqrt{{E_{\%\Delta,p0}}^2 + {E_{\%\Delta,p24|48}}^2} * 100$$

$$E_{\%\Delta,p0} = \frac{\partial}{\partial p_0}\%\Delta\delta_{p0} = \frac{-p_{24|48}}{p_0^2}\delta_{p_0}$$

$$E_{\%\Delta,p_{24|48}} = \frac{\partial}{\partial p_{24|48}}\%\Delta\delta_{p_{24|48}} = \frac{(1-p_0)}{p_0}\delta_{p_{24|48}}$$

$$E_{\%\Delta} = \sqrt{\left(\frac{-p_{24|48}}{p_0^2}\delta_{p_0}\right)^2 + \left(\frac{(1-p_0)}{p_0}\delta_{p_{24|48}}\right)^2} \times 100$$

Results

In the first trial, the control sample grew 42% after 24 hours. Here, the treatment groups actually grew well after 24 hours having a percentage increase of 95%, 96%, and 43% respectively. After 28 hours, the control group had grown very well with a 207% increase while the treatment groups were floundering with an overall growth of 6%, 10% and 21%. In the second trial, the overall growth of all samples is worse than that of the first trial. However, the control group does grow better than the treatment groups. After 24 hours, the control groups increased by 249% while the treatment groups grew by 154%, 50%, and 96%. After 48 hours, the control had grown by 60% overall, while the treatment groups had less parasites than they started with. The overall growth in the treatment groups was -13, -84%, and -41% respectively.

While the growth rates of the different treatment groups (with different magnetic field intensity) do not significantly differ from themselves, they are significantly lower than the control group at the 48-hour mark of both trials. The treatment is able to significantly inhibit growth of *Plasmodium falciparum*. However, when the magnetic field strength is increased, the effect is not visibly enhanced.

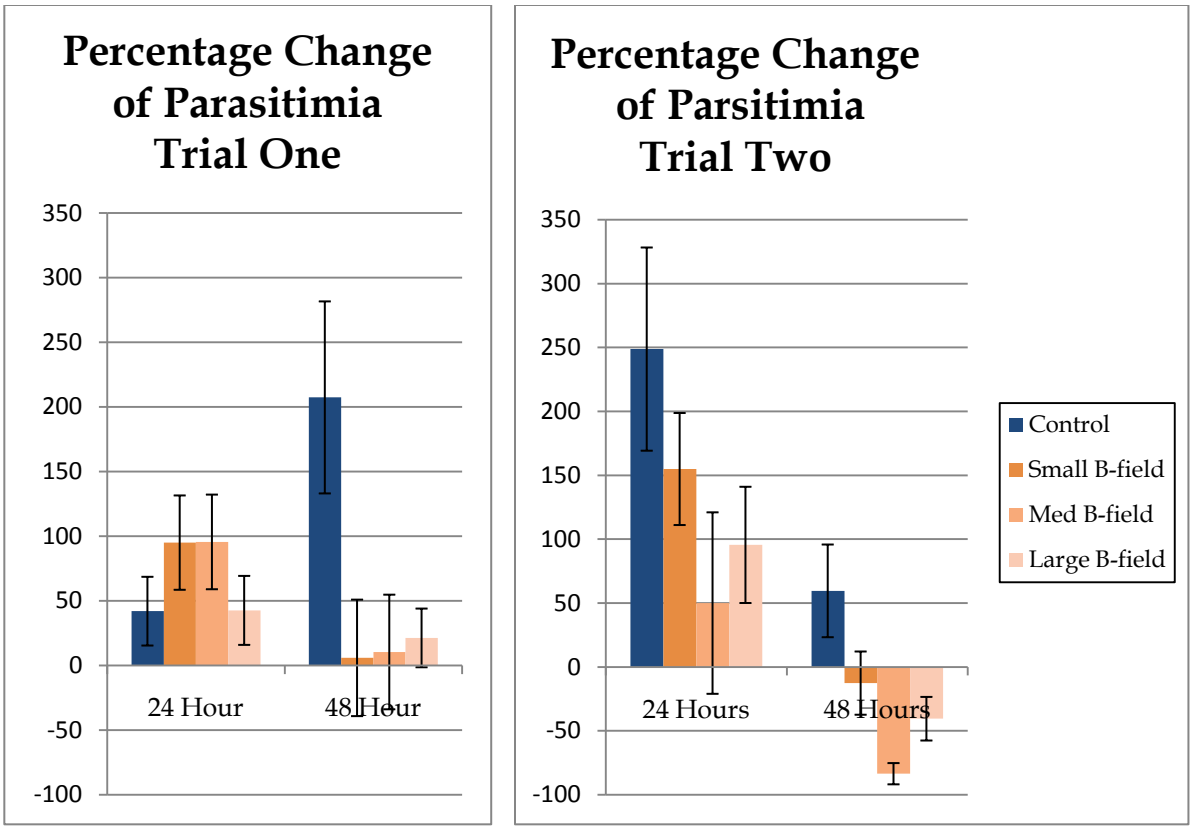


Figure 3 and 4 percentage change from 0 to 24 hours and from 48 hours to 48 hours. The blue marks he growth of he control. The three orange bars mark the three treatment groups with 0.682mT, 2.73mT, and 6.82mT root square mean applied field.

After 24 hours, in trial one, the increase in parasitemia is mainly in an increase of ring stage parasites. There are proportionally less late stage parasites in the treatment group than in the control group of trial one. In trial two , there are proportionally less late stage parasites in the treatment groups than in the control.

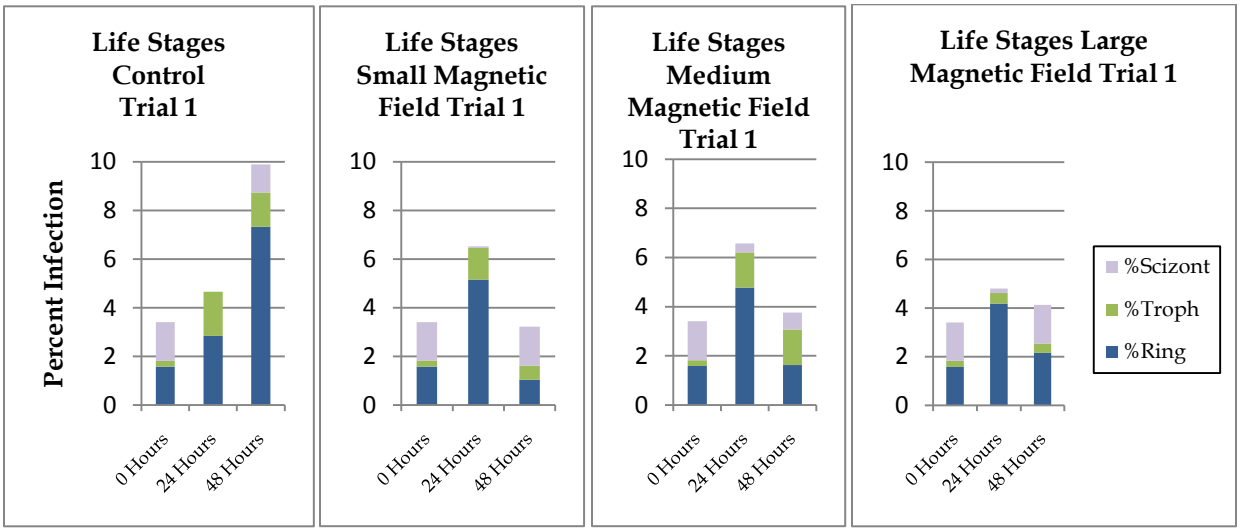


Figure 5,6,7 and 8. the percentage red blood cells infected by ring, trophozoites, or schizonts at 0 hours, 24 hours and 48 hours of treatment during trial one.

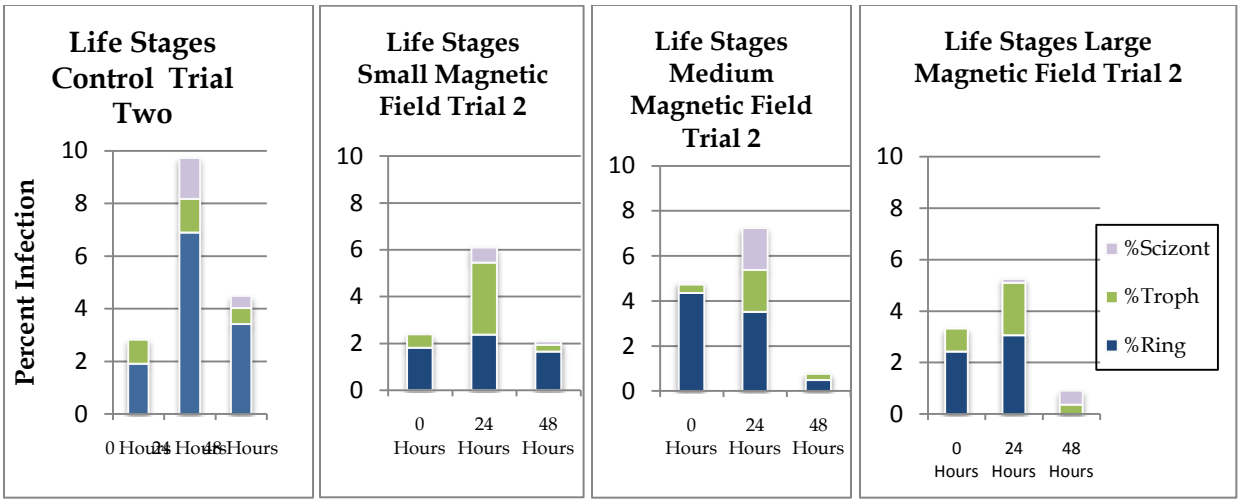


Figure 9,10,11 and 12. the percentage red blood cells infected by ring, trophozoites, or schizonts at 0 hours, 24 hours and 48 hours of treatment during trial two.

Conclusions

The treatment of weak alternating magnetic field significantly inhibits the growth of *Plasmodium falciparum*. In both trials, the growth of the cell cultures is much less than the control. In the second trail, there was some background effect that caused everything to not grow as well as it did in the first trail. However, the treatment groups always grew significantly less than the control group. The study of parasite change in each life cycle shows that the magnetic treatment affects the life stages differently. Presumably this is because the larger concentration of hemozoin a cell has the more susceptible it is to the effects of a magnetic field.

Future Directions

The mechanisms are not well understood. It is possible that a hemozoin crystal can feel a torque of an applied magnetic field killing the parasite. An experiment needs to be designed to test this theory. The effect of the magnetic field on the various life stages should also be examined. This can be accomplished by beginning the treatment with synchronize samples. In other words, samples will be treated starting with all rings, all trophozoites, and all schizonts

respectively. This work has already been started and will be continues this subsequent year. Different frequencies need to be tested; 5Hz was chosen arbitrarily by the previous research group. This parameter was kept the same in this experiment because it was shown to work. However, hopefully a different frequency can be found that will optimize the inhibition of *Plasmodium falciparum* growth. Using a cooling fan solved our ohmic heating problem by reducing an increase of 5°C to less than 1°C. However, the design needs to be improved to reduce further temperature variance between samples. The long term goal of this research is to develop a noninvasive treatment of malaria using weak alternating magnetic fields.

Additionally, the circuit design could be improved if a capacitor was added. If the correct capacitor was added so that the circuit's inductance was in resonance with the imputed current, we would be able to draw more voltage and have a more powerful and efficient magnetic field.

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