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Malaria: Not just a case of bad air

by

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Abbreviations

DMPO- Dimethyl pyrroline N-oxide
DTCS- N-dithiocarboxysarcosine
EPR- Electron paramagnetic resonance
HRP- Histidine rich protein
MGD- N-methyl-D-glucamine dithiocarbamate
nNOS- Neuronal nitric oxide synthase
RBC- Red blood cell

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Abstract

Malaria is a disease that affects millions of people, usually in Third World countries where effective treatments are expensive and rare. Malaria is a parasite spread by mosquitoes that breaks down hemoglobin in red blood cells. Heme is a toxic byproduct that is stored as hemozoin, an amorphous solid. Artemisinin and related compounds can be activated by hemozoin, effectively treating the malarial infection. Free radicals generated by the interaction of hemozoin and artemisinin cause oxidative stress, which destroys the invading parasite. This leads to the hypothesis that the malarial infection does not involve free radicals. This hypothesis can be tested by EPR imaging as well as checks using Electron Microscopy while manipulating levels of regulatory antioxidants.

Introduction

Malaria, from Italian for "bad air", is a disease that has plagued mankind for thousands of years. It is prevalent in Third World countries, especially near the tropics, where effective treatments are expensive and rare. Malaria affects 200-500 million people every year. Roughly 5% of all cases are fatal, making malaria second only to AIDS as Africa's most deadly disease [1]. Human malaria arises from infection by any of four species of protozoa of the genus *Plasmodium*: *vivax, ovale, malariae*, and *falciparum. P. vivax* is the most common, while *P. falciparum* is the most deadly [2].

The life cycle of the malaria parasite is very complex, with separate stages requiring transfer from mosquito to human and back for full reproduction. The life cycle starts when larvae, accumulated in the salivary glands of an *Anopheles* mosquito, enter a human when the mosquito bites. The parasite travels directly to the liver for the first stage of its life. It later matures and ventures out through the bloodstream, where it catabolizes red blood cells for protein, and discards the heme groups as hemozoin, also called hematin. Finally, the parasite is transferred back to another mosquito when it bites the human, and new larvae are created in the gut of the mosquito, which migrate to the salivary glands to start the cycle all over again.

Figure 1 introduces different drugs, which are used to treat the malaria parasite at different stages. While there is now widespread resistance to all these drugs, derivatives of each are still used frequently as treatments for malaria. Quinine is thought to increase the pH of the nucleus, disturbing DNA replication and attaching to the nuclear membrane, which disrupts signal transduction [3]. Chloroquine inhibits Histidine-rich protein, which is responsible for heme aggregation [5]. Artemisinin is activated by hemozoin, and the products of that reaction cause free radical reactions which lead to

alkylation of membrane thiols and the generation of high valent Fe-oxo compounds [4]. Malaria is adversely affected by both the failure of chloroquine-induced HRP and the radical products generated by artemisinin, so I hypothesize free radicals are not involved in the mechanism of malarial infection. This can be tested by several methods, such as EPR imaging of blood and liver cells upon infection, as well as looking for as well as checking the lifetime of invading cells when regulatory antioxidant levels are varied.

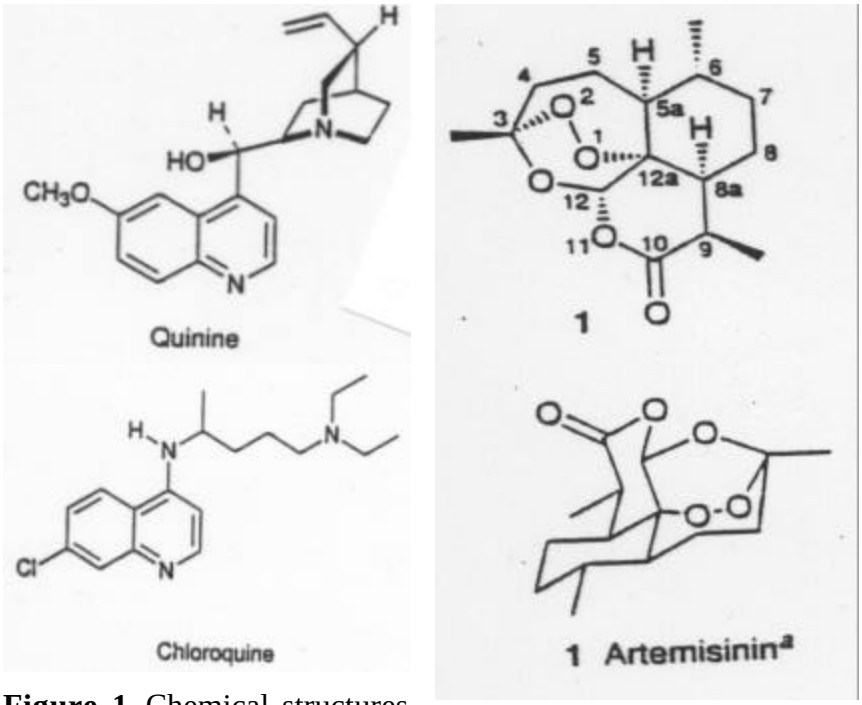
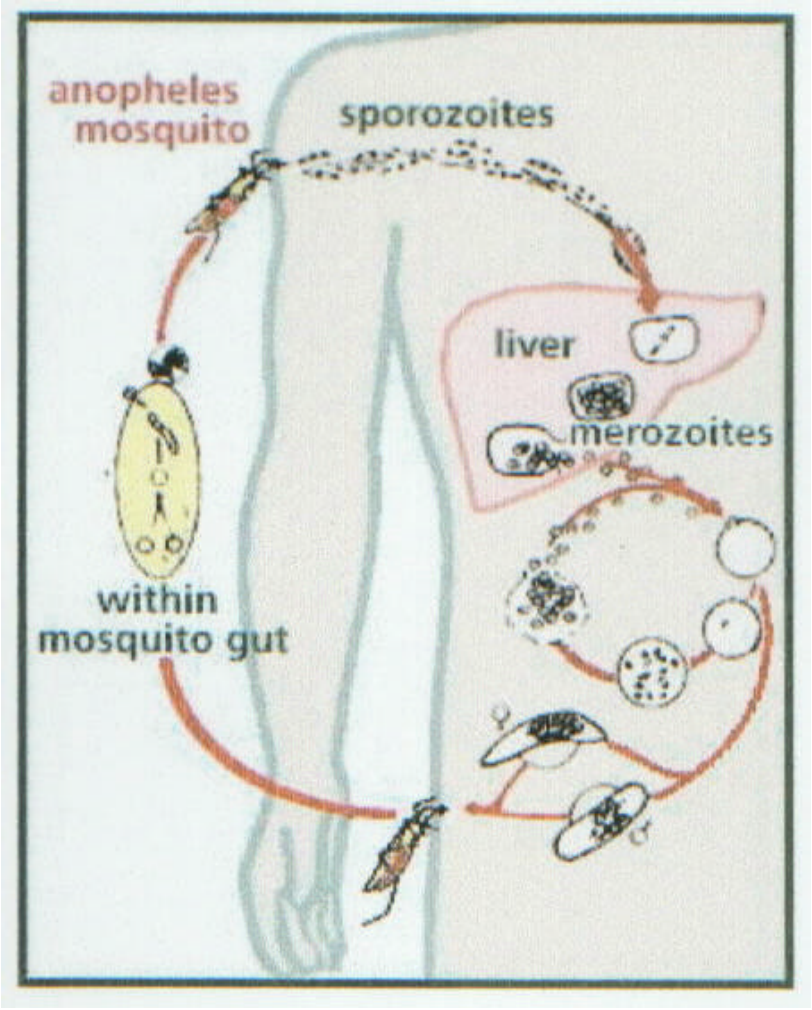


Figure 1- Chemical structures of common antimalarial drugs quinine, chloroquine, and artemisinin. Artemisinin is shown in 2 views to illustrate the 3-dimensional quality of the trioxane moiety. Adapted from [4].

Mechanism of Malarial Infection

A more detailed explanation of the mechanism of malarial infection is picture below in Cartoon 1. The disease is transmitted to humans through the salivary glands of an infected female *Anopheles* mosquito when the mosquito bites. Young forms of the parasite, called sporozoites, quickly travel to through the bloodstream and develop in the liver. After maturing for 1-2 weeks into the merozoite form, the liver cells rupture and

invade the red blood cells of the host. Once there, the parasite has two pathways. First, it can differentiate into gametocytes and asexually reproduce as many as 30 merozoites, which feed on the hemoglobin-rich environment of the red blood cell. After 2-3 days, the RBC bursts, releasing merozoites into the bloodstream and perpetuating the infection. This cyclical lysis of RBCs is responsible for the periodic fever, chills and headache commonly associated with malaria [7]. Second, the mature gametocytes remain in the RBCs of the host, and wait until it is again bitten by an *Anopheles* mosquito. The gametes reproduce sporozoites in the gut of the mosquito, which incubate in the salivary glands and start the process anew [8].



Cartoon 1- A pictorial representation of the life cycle of the malaria parasite, as described above. Adapted from [6].

While in the red blood cell, the parasite catabolizes hemoglobin, using the amino acids as a source of protein and aggregating the heme as hemozoin, a waste product. The structure of hemozoin was recently discovered, and is pictured below in Figure 2 [9].

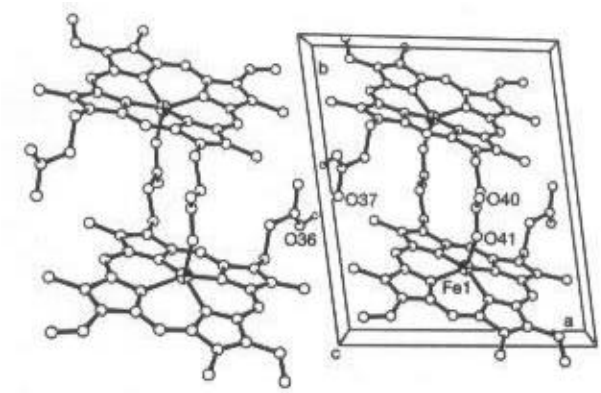


Figure 2- The structure of hemozoin. It is a polymer, non-covalently linked by two different bonds formed by the proprionates of protoporphyrin IX. One bond is formed between the iron center of heme and a proprionate, and the other is a hydrogen bond shared between proprionates. Adapted from [9].

Malarial treatments

Many treatments have been used to fight malaria in the last several thousand years. The Romans came up with the first treatment around the time of Christ. They realized mosquitoes passed on the disease, so they drained swamps to get rid of the mosquito population and remove the vector for malarial infection. This was the only treatment for approximately 1700 years, so humans evolved a defense mechanism, sickle cell anemia. This is a non-lethal mutation that prevents malarial infection, but the side effects are very undesirable. To this day, most victims of sickle cell anemia originally lived in the tropics, where malaria was prevalent. Natural products have also been used to treat malaria. In the 17th century, western explorers discovered Incans in Peru who chewed the bark of the "Fever Tree" to treat malaria. In 1820, two French pharmacists, Pelletier and Caventou isolated the two main alkyloids responsible for treatment, quinine and cinchonine from the *Cinchona* tree.

Quinine was used until 1930 as the sole treatment for malarial infection, when derivatives, such as chloroquine, mefloquine, and amodiaquine, with fewer side effects

were introduced. Unfortunately, resistance to the derivatives sprung up quickly. In fact, resistance has made chloroquine useless in several parts of the world. So other methods of malaria treatment needed to be found.

In 1967, the Chinese government commissioned a study to examine the validity of alternative, holistic treatments. Researchers examined ancient texts describing medicinal purifications from natural products, and found a treatment for a "periodic fever". The antipyretic activity of the leaves of *Artemisia annua* was first written down as early as 340 A.D., and its antimalarial activity was noted in 1596. In 1972, a low temperature extraction yielded a crystalline compound they called qinghaosu, which was later renamed artemisinin. Artemisinin was effective at treating malaria, but derivatives such as artemether and arteether are more potent, and are used today in several Third World countries [4]. Interestingly, the US FDA has not approved artemisinin or its derivatives for treatment of malaria, but this is probably because malarial infections in the United States are so rare that normal quinine-based treatments are effective.

The mechanisms of these treatments work on slightly different stages of the malaria life cycle, but a couple utilize the properties of hemozoin. When chloroquine is effective as a treatment, it inhibits HRP, the protein responsible for heme aggregation. Artemisinin is activated by hemozoin, as seen in Figure 3.

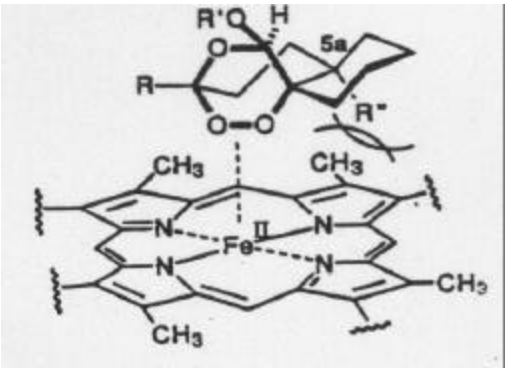


Figure 3- Binding of artemisinin to heme. The high energy O-O bond is cleaved by the Fe of heme, resulting in the generation of highly reactive species. Notice the overlap of the R'' with the 1- and 2-positions of the heme. This interaction is used to "tune" the drug for the specific type of malarial infection. Adapted from [10].

An understanding of the mechanism of artemisinin treatment is essential to prove my hypothesis, so it will be examined in detail. Free radicals generated by artemisinin treatment have been trapped *in vitro*, as shown in Figure 4.

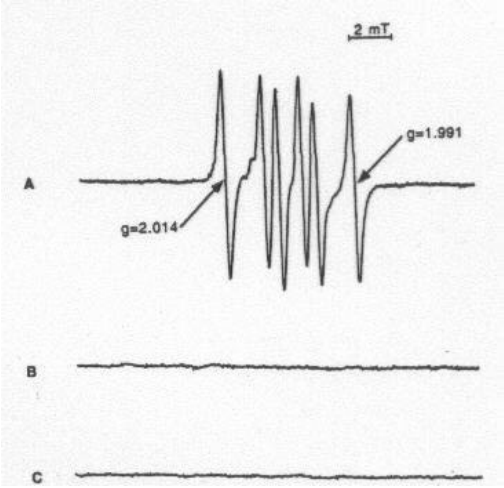
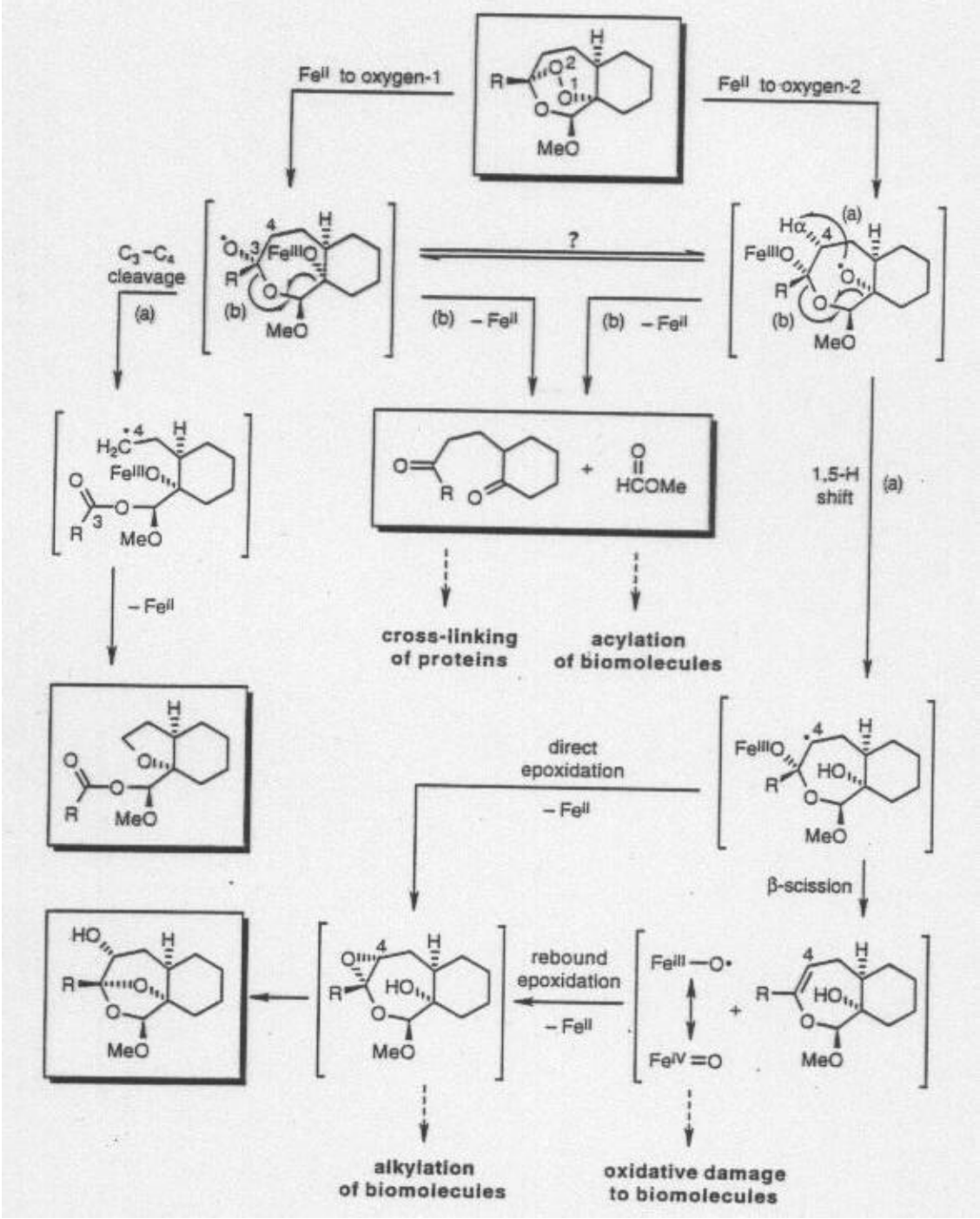


Figure 4- EPR spectra of A) artemisinin, iron, and DMPO, B) artemisinin and DMPO, and C) iron and DMPO. Adapted from [11].

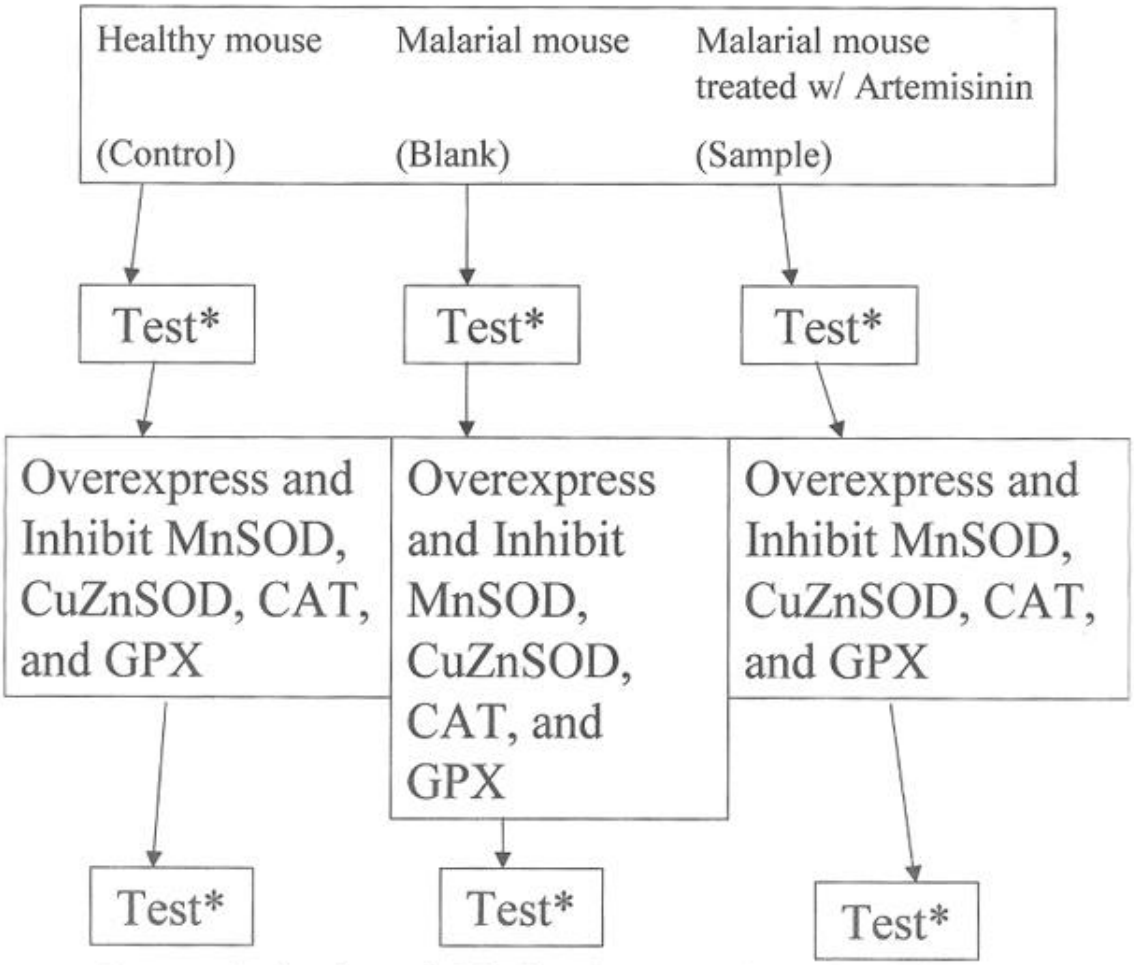
The mechanism of artemisinin is very complex, due to the reactive nature of the species generated. It can cause damage to the cell in several ways, by cross linking proteins, acylating or alkylating biomolecules, or causing general oxidative damage to membranes. All of these mechanisms are succinctly summed up in Scheme 1, on the following page.

The behavior of the parasite upon these treatments led to the formation of my hypothesis, that free radicals are not involved in the mechanism of malarial infection. Hemozoin is a waste product for a reason, and that reason is that the free radical reactions it catalyzes are toxic to the cell. The parasite lacks the antioxidant system used by higher cells, so I hypothesize free radicals are not involved in malarial infection.



Scheme 1- The various damage causing pathways of artemisinin. The free radicals and reactive species generated wreak havoc on the parasite. I believe this is because the parasite has no mechanism set up to deal with free radicals, because free radicals are not involved in the pathology of the disease. Adapted from [12].

Hypothesis: Malaria does not involve free radicals



Data analysis: Control-Blank = Answer to Does Malaria involve free radicals?

Sample-Blank = Answer to Are free radicals generated only upon treatment?

Test*= EPR imaging of both liver and blood, check level of oxidation by 4HNE in blood vessels and 8HG in DNA

Proving the Hypothesis

The flow chart on the preceding page lays out the basic tests that will lead to the approval or disproval of my hypothesis. A mouse is a valid candidate to study for many reasons. A mouse infected with the murine version of malaria, *P. berghei*, reacts similarly to a human infected with *P. falciparum*, the most lethal type of malaria. The small size of a mouse is necessary for EPR imaging of its liver. This must be done while it is alive, so the sample chamber has to be big enough for the mouse to fit. In addition, genes will be expressed and inhibited, and their effects monitored. This has been done in the past.

The scientific method will be used to prove or disprove my hypothesis. A healthy mouse must be measured to set up a control for the experiment. Next, a mouse with malaria will be measured as a blank. A subtraction of Blank-Control will tell if free radicals are involved in malarial infection. The sample mouse will be infected with malaria, and then treated with artemisinin. A subtraction of Sample-Blank will tell how many free radicals are generated upon treatment. I postulate the answer to the former equation will be close to zero, but the answer to the latter will be several orders of magnitude greater.

Tests

Many of the tests that will be performed have been done in some fashion in the recent past. For example, EPR imaging has been used to look at free radical generation in murine cardiac tissue [13], but in this instance liver tissue will be examined. EPR spectra of RBCs of rats have been examined [14], but in this case RBCs of mice will be

examined. Therefore, all the experiments proposed have a valid scientific precedent, and should help determine the validity of the hypothesis.

The first test done will tell the most about the mechanism of the disease. It will be to examine the liver of healthy, infected, and treated mice using EPR imaging techniques. While no nitric oxide species are known to form directly from the treatment of malaria with artemisinin, they are a good indicator of radical reactions. Endogenously produced nitric oxide radicals can be trapped using DTCS and MGD, whose structures are pictured below, in Figure 5.

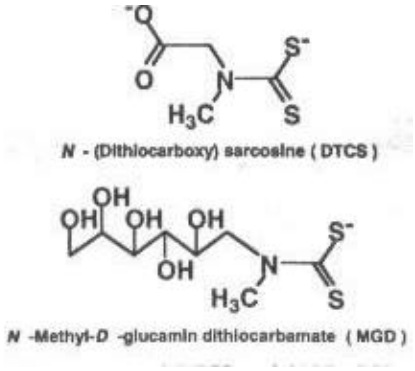


Figure 5- Spin traps used to detect the presence of NO in living organisms. These compounds complex with iron before combining with NO. Adapted from [15].

These spectra can be collected at room temperature with an S-band microwave bridge operating at 3.5 GHz.

If this method does not work, analysis of the blood and other tissues is also possible. The method used by Trentz, et al to examine rat hemoglobin during shock syndrome could be applied [16]. They utilized a clamping method to pinch off important sections of lung tissue. This procedure could work with liver tissue for these experiments. They also used a fast-freezing technique to draw blood from the jugular vein of a rat. This could be useful for the next experiment.

Mason, et al have examined RBCs for free radical content after endotoxic shock induced by carbon tetrachloride [14]. They saw a signals at g = 2.48, 2.29, and 1.91,

which were attributed to various breakdown products of cytochrome P420. The spectra are shown below in Figure 6.

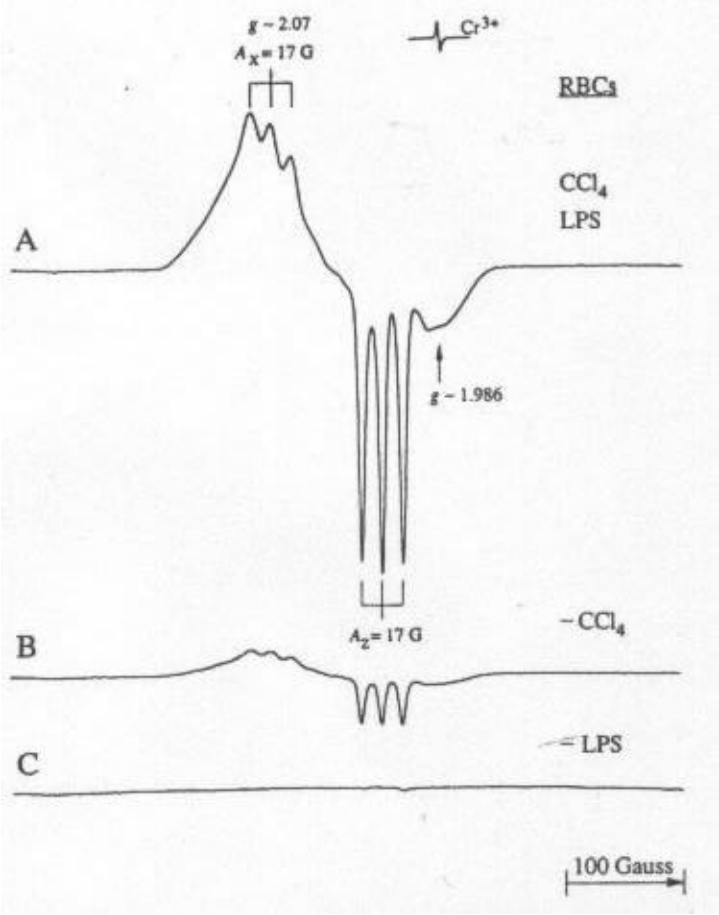


Figure 6- EPR spectra measured at 77K from rat RBCs. A) Rat injected with CCl_4 and then with an activating factor 20 hours later. The spectrum was taken 4 hours after the second injection. B) CCl_4 replaced with olive oil. C) Activating factor replaced with saline. Spectrometer conditions were as follows: modulation amplitude, 4 G, Microwave power 10 mW, time constant 1.3 sec, scan rate 49 G/min. Adapted from [14].

A series of these tests performed on control, blank, and sample mice should give different results, if my hypothesis is correct. The control mouse should have little free radical activity; in fact any activity will be set up as a baseline. The blank mouse will probably have slightly more free radical activity due to the infection of malaria, but not significantly so. Finally, the sample mouse should have several times more free radical activity than any of the others.

The next series of tests involves expression and inhibition of antioxidants. This will radically alter the chemistry occurring in the infected RBCs. The effects could be checked by blood counts for invading cells using Electron Microscopy, or by using the

EPR methods mentioned above. For example, if CuZnSOD is overexpressed, it will produce extra H_2O_2. This extra peroxide will react with hemozoin, yielding Fenton chemistry and the highly reactive hydroxyl radicals that accompany it. I speculate this would be devastating for the malarial parasite, because it doesn't have the advanced antioxidant systems that higher cells do. If malarial infection is not accompanied by free radical reactions, large amounts of extraneous free radicals will cause severe damage to the parasite.

The liver contains both MnSOD and CuZnSOD, so both would be expressed and inhibited. Overexpression has already been studied in the control mouse, and would have the effect of lengthening the mouse's life as well as other commonly known effects. Since protozoans do not contain CuZnSOD [17], its overexpression may cause problems because the cell will not be set up to deal with all the H_2O_2 produced. When both SODs are inhibited, however, I predict the parasite will be destroyed easier and quicker than normal. If superoxide is allowed to remain longer in the cell, it will oxidize fatty acids and cause problems all over. Higher cells have backup measures set up to deal with this, and while the cells would age faster and die a little quicker, the organism as a whole would survive. I think the parasite would not be as well prepared. In the sample mouse, the large number of free radicals generated by artemisinin would increase its toxicity to the invading parasite. The normal effect of the drug could probably be obtained with a lower dosage.

If CAT is inhibited while both SODs are overexpressed, the effect would probably be worse for both cell types. Once again, I predict the higher cells would survive, while the malarial cells would die quite easily. If CAT is inhibited and all other concentrations stay normal, the effect would be detrimental to the parasite, but probably

nonlethal. I think the inhibition of CAT would have a large effect on the sample mouse, because one of its products, O_2, will interact with the radicals produced by artemisinin to form superoxide. If SOD is inhibited at the same time, this will prove to be toxic to both cell lines. If SOD is normal, I predict little change in activity.

Finally, the manipulation of GPx would also greatly affect the cells. In the control, GPx inhibition would cause proteins to be in incorrect oxidation states, and therefore conformational states, so several problems would result. The overexpression of GPx wouldn't be as severe. In the blank mouse, similar results would be expected. A dramatic effect would be seen in the sample mouse. Since artemisinin oxidizes thiols, inhibition of GPx would be definitely be toxic to malarial cells, and probably toxic to higher cells as well. A similar effect to SOD inhibition would probably be seen; a lower dosage would be as effective. The manipulation of these regulatory enzymes will all cause significant changes to the cell. If the scientific method is used, with a rigorous system of controls and samples, each individual effect could be studied and noted.

Conclusions

Malarial infection involves a complex cycle, which probably doesn't involve free radical reactions. This hypothesis can be tested using a mix of EPR imaging and cell counting upon the expression and inhibition of regulatory antioxidant enzymes. A rigorous adherence to the scientific method would be necessary to control all variables, but this project is viable.

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