ANTI-MICROBIAL PHOTODYNAMIC THERAPY

Mechanisms of action

Antimicrobial Photodynamic Therapy is a treatment option based on the combination of a photosensitizer that is selectively localized in the target tissue and the intravenous application of light of appropriate wavelength to activate the photosensitizer, resulting in photodamage and cell death. Exposure of the photosensitizer to light of a specific wavelength will lead to light absorption by the photosensitizer, thereby lifting it from a short-lived (nanoseconds) state to an excited singlet electronic state. The singlet-state photosensitizer can then undergo an electronic transition (spin flip) to a much longer-lived (microseconds) triplet state. The longer lifetime allows the triplet PS to react with ambient (ground state) oxygen by one of two different photochemical pathways, called Type 1 and Type 2 photochemical pathway. Type 1 involves an electron transfer to produce superoxide radicals and then hydroxyl radicals, while Type 2 involves energy transfer to produce excited state singlet oxygen. Superoxide, hydroxyl radicals and excited state singlet oxygen are highly reactive oxygen species (ROS) that can damage nearly all types of biomolecules (proteins, lipids and nucleic acids) and kill cells by inducing irreparable oxidative damages. These processes are called photodamage. Typical examples for those damages are the inhibition of protein synthesis and molecular alterations of DNA strands through DNA-protein cross linking or strand breaks. These processes alter the transcription of the genetic material during its replication (mutagenic effect) and finally lead to microbial death.

The killing of microbial cells via aPDT is rapid and only takes few seconds (while the action of antibiotics can take hours or days).

History and State-of-the-Art

Against the background of increasing pathogen resistances against antibiotics, laser-therapy is just about to move back to the spotlight after its efficacy to treat infectious diseases has been known for more than a hundred years already. The previous negligence of the therapy has to main reasons:

Firstly, the first breakthroughs in research in the 1940s were achieved simultaneously to the development of the first commercially available antibiotics (e.g. Penicillin). In reaction to that, research funds for aPDT had been reduced radically. Since a couple of years, this trend is reversed as aPDT becomes more interesting again in times of increasing pathogen resistances against antibiotics. In this context it should also be mentioned that, a broad range of studies shows that aPDT is equally effective against multi- resistant pathogens than it is against non-multi- resistant pathogens. Further, all evidence suggests, that pathogens will not be able to develop resistances to aPDT in the future.

In addition to that, one of the main advantages of aPDT towards other treatment options such as antibiotics include that it is applicable for a large variety of pathogens and not only for bacteria.

The probably even more important reason for the previous negligence of aPDT is secondly that until very recently physicians weren´t able “to take aPDT out of the labour”. The limiting factor had been the disability of science to activate the photosensitizers within the body with light of appropriate wavelength.

Nowadays, the methods of intravenous blood irradiation and interstitial laser therapy facilitate the treatment. Photosensitizers can be activated intravenously if bound to pathogens present in the blood stream or interstitially if bound to locally occurring pathogens.

Thereby, photosensitizers have to comply with the following requirements.

Selection criteria for aPDT photosensitizers

Pathogen specific photosensitizers for many major tropical diseases have been known from various in-vitro studies for years.

More or less analogous to the selection criteria for photosensitizers for photodynamic tumour therapies (introduced in chapter 7.4), a successful photochemical technology that will eradicate pathogens requires that the perfect sensitizer has the following properties:

- Firstly, the ideal sensitizer must bind to pathogenic particles but must not bind to plasma proteins, platelets or red blood cells. If the sensitizer binds only to pathogen, in the presence of plasma protein and platelets, radiant energy in the UVA or visible region of the spectrum can be deposited selectively into the pathogen, even in the presence of blood components. The ideal sensitizer must therefore recognize and exploit a chemical difference between the pathogen and the blood product. In this context, one differentiating factor between pathogens and blood components is crucial: Bacteria and many types of virus are encapsulated by phospholipid membranes or envelopes, but blood components are not.
- Secondly, the ideal sensitizer should be non-toxic, non-mutagenic and must not break down to compounds that are toxic and mutagenic. It should be readily available, water soluble, and inexpensive.
- Finally, absorption of radiation must produce a short-lived, highly reactive toxin, which creates lesions in its immediate vicinity (e.g., only to the pathogenic particle to which it is bound).

One of the photosensitizers that already proofed to be effective in aPDT is Hypericin. It has anti- bacterial and anti- viral effects and is used successfully to treat lyme disease and other viral infections.

Riboflavin as the photosensitizer of choice

Riboflavin fulfils all the above-mentioned criteria for an adequate PS. It selectively binds to DNA (Dardare et al) and can be photo-excited with low energy visible light of wavelength in the UV- or blue-light-spectrum with a preferred absorption peak at 447 nm. It also is naturally presented in the human body and not toxic or mutagenic. After activation with light it causes significant damage to nucleic acid without affecting plasma proteins, erythrocytes or platelets (but as it binds to nucleic acid, damage at leucocytes may occur. However, these effects were neglectable in all clinical studies). Riboflavin is thus under study as a sensitizer of nucleic acid damage in the presence of other biological chromophores.

General Description

Riboflavin (RB, vitamin B_2) is a vitamin essential to the human diet. It is present in aerobic organisms and is found in many foodstuffs such as milk, beer, eggs, yeast and leafy vegetables. It is also the precursor for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are major coenzymes that participate in a number of one-electron processes in the human body.

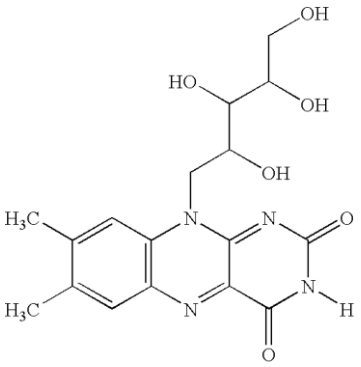

Figure 1: Molecular structure of Riboflavin

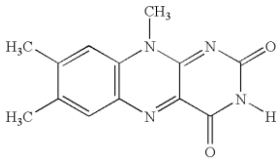

Figure 2: Molecular structure of Lumiflavin (LF)

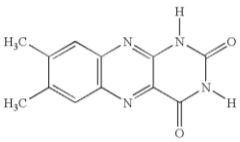

Figure 3: Molecular structure of Lumichrome (LC)

Riboflavin has absorption maxima at 220, 265, 375, and 447 nm in water and is yellow-orange in color. When aqueous solutions containing riboflavin are exposed to sunlight, riboflavin is converted into lumichrome (LC) under neutral conditions, and into lumiflavin (LF) in alkaline solutions. LC is also a known metabolic breakdown product of riboflavin in the human body.

Advantages of using riboflavin as a sensitizer include the fact that riboflavin is 'Generally Regarded As Safe' or GRAS by the FDA. Its photochemical breakdown products (lumichrome and lumiflavin) are metabolites of riboflavin and thus are formed naturally in vivo.

Besides being non- toxic and non- mutagenic and an absorption spectrum outside the range of light that is strongly absorbed by plasma proteins or platelets, Riboflavin has another property that makes it attractive as a photosensitizer in aPDT: Platelets treated with riboflavin and light continue circulating normally even under conditions which inactivate high titers of pathogen.

Thus, technology based on an endogenous sensitizer like riboflavin is much safer than technology based on a synthetic sensitizer (e.g. a psoralen or methylene blue). Riboflavin also has the advantage over other possible synthetic photosensitizers because it is a naturally occurring, essential vitamin with no known toxicity.

In-Vitro Data: Anti-microbial Photodynamic Therapy with Riboflavin (with MIRASOL system)

Goodrich et al. have developed the so called Mirasol Pathogen Reduction Technology (PRT) system in which Riboflavin and ultra-violet light are used to reduce pathogen loads in blood products. In this context, they have conducted several in-vitro studies to determine the effects of the treatment on pathogen reduction. Their results (see following tables) are very promising for a wide range of different pathogens and their system is already established in transfusion medicine.

Virus	Model virus used	Log/ml reduction [a]	Type
HIV, latent	intracellular human	4.5	Enveloped
HIV, active	cell-associated human HIV	5.9	Enveloped
West Nile virus	West Nile virus	≥5.1	Enveloped
Hepatitis C virus	sindbis virus	3.2	Enveloped
Hepatitis B virus	pseudorabies virus	2.5	Enveloped
Rabies virus	vesicular stomatitis virus	≥6.3	Enveloped
Influenza virus, avian flu virus	influenza A virus	≥5.0	Enveloped
Cytomegalovirus	infectious bovine rhinotracheitis virus	2.1	Enveloped
Human B19 virus	porcine parvovirus	≥5.0	Non-enveloped
Hepatitis A virus	human hepatitis A	1.8	Non-enveloped
Hepatitis A virus	encephalomyocarditis virus	3.2	Non-enveloped
Chikungunya virus	La Reunion clinical isolate	2.1	Enveloped
[a] Results expressed as '≥' indicate that the pathogen load was reduced to the limit of detection of the assay.			

Figure 4: Virus log reduction results from standard in vitro assays for infectivity (TCID50) [Goodrich et al. 2011]

Disease	Parasite	Log / ml reduction [a]
Leishmaniasis	Leishmania donovani infantum	≥4.0
Malaria	Plasmodium falciparum	≥3.2
Chagas disease	Trypanosoma cruzi	≥5.0
Babesiosis	Babesia microti	≥4.0
Scrub typhus	Orienta tsutsugamushi	≥5.0
[a] Results expressed as '≥' indicate that the pathogen load was reduced to the limit of detection of the assay.		

Figure 5: Parasite log reduction results from standard in vitro assays for infectivity or bio-assay in hamsters [Goodrich et al. 2011]

Bacteria	Type	Occurrence	Mirasol % effectiveness	Culture method % effectiveness
Staphylococcus epidermidis	Gram-positive	20	100	27
Escherichia coli	Gram-negative	8	100	100
Bacillus cereus	Gram-positive	7	100	100
Staphylococcus aureus	Gram-positive	6	90	53
Streptococcus agalactiae	Gram-positive	5	100	100

Streptococcus mitis	Gram-positive	5	100	100
Streptococcus pyogenes	Gram-positive	5	100	100
Enterobacter cloacae	Gram-negative	4	100	100
Propionibacterium acnes	Gram-positive	3	100	0
Serratia marcescens	Gram-negative	3	100	100
Klebsiella pneumoniae	Gram-negative	2	100	100
Acinetobacter baumannii	Gram-negative	1	66	100
Yersinia enterocolitica	Gram-negative	1	100	100
Overall % effectiveness			98	66
[a] Occurrence is the number of cases of contaminated platelets with the particular bacteria as reported in hemovigilance studies[33]. Percent effectiveness is the ability to inactivate or detect the particular bacterial strain at initial contamination levels of < 100 CFU per product. Overall effectiveness is the multiple of the percent effectiveness with the frequency of occurrence for this agent [34].				

Figure 6: Inactivation of bacteria [a]

In-Vivo Data: Malaria-Pilot-Study in Ondo (Nigeria) on Plasmodium Falsiparum (2017)

Evaluation of anti- microbial photodynamic therapy (using riboflavin as sensitizer) as a safe and effective treatment option for Malaria

By Robert Weber, Martin Junggebauer, Dr. Habeeb Ali

Objective: Evaluation of anti- microbial photodynamic therapy (using riboflavin as sensitizer) as a safe and effective treatment option for Malaria.

Method: Usage of Riboflavin as photosensitizer. Activation by intravenous application of blue light with a wavelength of 447nm. Subsequent intravenous laser therapy with green, yellow and red light.

Design: Comparison between treatment and control group, both composed of patients suffering from Malaria Falsiparum. The treatment group received aPDT treatments plus intravenous laser therapy, the control group conventional therapy only.

Results: No adverse effects on blood components. Effective parasite killing. Quick improvement of presented symptoms.

Conclusion: The applied protocol seems to be a safe and effective treatment option for Malaria.

Malaria as target-disease for Anti-Microbial Photodynamic Therapy

Malaria is an infectious disease of humans and other animals that is transmitted by mosquitos and is caused by parasitic protozoans and belongs to the genus Plasmodium. There are several infective members of the Plasmodium genus. The most serious is P. falciparum, less serious forms are P. ovale, P. vivax and P. malariae. Relapsing fevers are often encountered with infection by P. knowlesi.

All various forms of Malaria assume many different physical forms during its life cycle. Within the scope of this study the bloodstream phase (erythrocytic phase) is the phase of interest.

During the bloodstream phase, various photosensitizers can be taken up by plasmodial cells inside erythrocytes while there is no specific uptake by uninfected erythrocytes. In this context, an effective irradiation of the blood circulation has long been the obstacle for the application of aPDT to Malaria patients. Since far more sophisticated technologies are available today aPDT can be applied to treat Malaria patients by attacking the parasites during their erythrocytic life cycle stage. The technology of choice at the moment is the Weberneedle Endolaser © system.

Study Design

The first pilot study was designed as a randomized controlled trial in which 20 adult outpatients suffering from the severest form of malaria - plasmodium falsiparum - were randomly selected and assigned equally into either the treatment or the control group. Consequently, both the treatment and the control group consisted of ten (10) patients each. Patients in the treatment group were exposed to five (5) sessions of aPDT over the course of 9 days while those in the control group received the conventional therapy only. The study was conducted at the Adeyemi College of Education clinic in Ondo, Nigeria.

Data was collected at the onset, midway and at the end of the study. Indicators such as time to malaria parasite clearance and time to disappearance of symptoms was closely monitored in both groups and compared at the end of the study.

Protocol

At first, a special Riboflavin IV solution was administered to the patients over a period of 30 minutes. The photosensitizer was allowed to accumulate at the DNA of the malaria parasites over a period of 30 minutes. This ensured that most unbound Riboflavin has been removed from the bloodstream to avoid photon uptake by the same.

Laser light was then applied directly to the blood stream by using the *Weberneedle Endolaser ©*. A 447nm blue laser with maximum output power of 100mW was applied at 100% for a period of 45 minutes to photoactivate the Riboflavin. This was followed by the application of green (532nm at 50% of 50mW max. output), yellow (589nm at 50% of 50mW max. output) and red (635nm at 35% of 100mW max. output) low-level-laser-light through the same catheter system for 10 minutes each.

Each patient in the treatment group received five sessions of this treatment regime within 9 days with an interval of one day in between the sessions. The patients were closely monitored and conventional therapy would have been given to them if their symptoms would not have improved after two treatment sessions.

Biomedical tests for malaria parasites were done on a daily basis after the second treatment session. On top of that, patients filled out a questionnaire after each treatment by which they were asked about the improvements

of their symptoms. To account for safety concerns haematocrit values and total white blood cells were measured in the patients before and after the therapy.

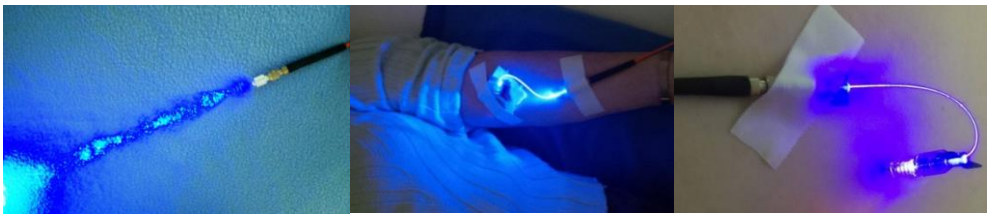

Figure 7: Application of 447 nm blue laser at 100% of 100 mW max. output (Weberneedle Endolaser ©)

Results

➔ **Safety: Biomedical tests**

Haematocrit values were gathered for all patients while white blood cells counts were gathered only for participants in the study group. The results are summarised in Figure 8.

There was a difference of 2.8±5.0 in the baseline and final haematocrit of patients in the control group while this difference was 2.1±1.2 for those in the study group.

The mean values of the white blood cell counts were 8020±1880, 7240±1820 and 7220±1250 for the baseline, midway and final examination respectively.

Student t – test statistic revealed no significant difference between the initial haematocrit (M=38.90, SD=4.20) and final haematocrit values (M=41.80, SD=7.16), t=1.299, p=0.250 in the control group. Likewise, the difference between initial (M=33.67, SD=4.09) and final (M=35.44, SD=0.94) haematocrit values among patients in the study group was not statistically significant (t=2.101, p=0.069). The results are illustrated in Figure 9below.

A comparison of white blood cell numbers using ANOVA showed no significant difference (F=5.433, p=0.097) in the white blood cells values before, during and after the therapy. The results are illustrated in Figure 10 below.

Put all together, these findings indicate that the applied treatment protocol is safe and did not go along with any adverse effects on blood components.

Test (mean of values)	**Time of observation**	**Mean values**	
		Control group	**Study group**
PCV	Baseline	38.9±4.2	33.3±4.02
	Final	41.8±7.2	35.4±2.8
WBC count	Baseline	-	8022±1882
	After third session	-	7244±1823
	Final	-	7222±1253

Figure 8: Haematocrit and WBC count

	PCV	Mean	SD	N	df	t	p
Control	Initial	38.90	4.20	10	9	1.229	0.250
	Final	41.8	7.16	10			
Study	Initial	33.67	4.09	9	8	2.101	0.069
	Final	35.44	2.83	9			

Figure 9: Statistical test of significance for haematocrit values

Source	df	SS(000)	MS(000)	F	p
Between groups	5	25535.56	5107.11	5.433	0.097
Within groups	3	2820.00	940.00		
Total	8	28355.56			

Figure 10: One – way analysis of variance for WBC counts in the study group

➔ **Treatment outcomes: Malaria parasite screening**

Using the rapid diagnostic test (RDT) kits, all patients were tested for the presence/absence of P. falciparum. Patients in the control group were tested before the first treatment took place and then again after the last treatment. Patients in the study group were tested before the first treatment took place, after the second, third and fourth treatment and lastly after the final therapeutic session.

The results are presented in Figure 11. In the control group 5 (50.0%) of the patients were still tested positive at the end of the treatment cycle. Significantly better results were observed in the study group where 8 out of 9 (1 drop out / 88,9%) patients were tested negative for Malaria at the end of the study.

Already after the third and fourth sessions, 2 (20.0%) resp. 4 (40.0%) of the patients in the study group were tested negative, indicating remarkably quick recovery processes.

Time of test	Test result	Control	Study
Baseline	Positive (%)	10(100.0)	10(100.0)
	Negative (%)	-	-
After T2	Positive (%)	-	10(100.0)
	Negative (%)	-	-
After T3	Positive (%)	-	8(80.0)
	Negative (%)	-	2(20.0)
After T4	Positive (%)	-	6(60.0)
	Negative (%)	-	4(40.0)
Final/after T5	Positive (%)	5(50.0)	1(11.1)

	Negative (%)	5(50.0)	8(88.9)
	Total (%)	10(100.0)	9(100.0)

Figure 11: Malaria parasite screening

➔ **Treatment outcomes: Symptoms**

The following tables (Figure 12 - Figure 15) show the symptoms presented by the patients throughout the study. While some symptoms persist in some of the patients in the control group even after treatment with ACT (regular Malaria drug), there was a gradual reduction in the number and severity of symptoms presented by those in the study group. Symptomatic improvement could be observed in all patients in the study group except for one (who decided to exit the study).

Symptoms	**Time of observation**	**Control group (%)**		**Study group (%)**	
Shaking chills	Baseline	Yes	6(60.0)	Yes	7(70.0)
		No	4(40.0)	No	3(30)
	After T2	-	-	Yes	2(20.0)
				No	8(80.0)
	After T3	-	-	Yes	-
		-	-	No	10(100.0)
	After T4	-	-	Yes	-
		-	-	No	10(100.0)
	Final	Yes	0(0.0)	No	9(100.0)
		No	10(100.0)	-	-
Fever	Baseline	Yes	10(10.0)	Yes	10(100.0)
		No	0(0.0)	-	-
	After T2	-	-	Yes	6(60.0)
		-	-	No	4(40.0)
	After T3	-	-	Yes	2(20.0)
		-	-	No	8(80.0)
	After T4	-	-	Yes	1(10.0)
		-	-	No	9(90.0)
	Final	Yes	4(40.0)	No	9(100.0)
		No	6(60.0)		
Fever severity	Baseline	Mild	2(40.0)	Mild	-
		Moderate	7(70.0)	Moderate	8(80.0)
		Severe	1(10.0)	Severe	2(20.0)
	After T2			Mild	3(30.0)
				Moderate	2(20.0)
				Severe	1(10.0)
	After T3			Mild	-
				Moderate	-
				Severe	-
	Final	Mild	4(100.0)		
	Baseline	Yes	1(10.0)	No	10(100.0)
		No	9(10.0)		

Profuse sweating	Final	No	10(100.0)		

Figure 12: Malaria symptoms presented - Part 1

Symptoms	Time of observation	Control group (%)		Study group (%)	
Headache	Baseline	Yes	10(100.0	Yes	10(100.0)
	After T2			Yes	10(100.0)
				No	-
	After T3			Yes	8(80.0)
				No	2(20.0)
	After T4			Yes	2(80.0)
				No	2(20.0)
	Final	Yes	5(50.0)		-
		No	5(50.0)	No	9(100.0)
Headache severity	Baseline	Mild	2(20.0)	Mild	-
		Moderate	5(50.0)	Moderate	6(60.0)
		Severe	3(30.0)	Severe	4(40.0)
	After T2			Mild	4(40.0)
				Moderate	5(50.0)
				Severe	1(10.0)
	After T3			Mild	8(100.0)
				Moderate	-
				Severe	-
	After T4			Mild	3(100.0)
				Moderate	-
				Severe	-
	Final	Mild	5(83.3)		-
		Moderate	1(16.7)		-
Nausea	Baseline	Yes	2(20.0)	Yes	5(50.0)
		No	8(80.0)	No	5(50.0)
	After T2		-	Yes	1(10.0)
			-	No	9(90.0)
	After T3		-	Yes	1(10.0)
			-	No	9(90.0)
	After T4		-	Yes	1(10.0)
			-	No	9(90.0)
	Final	No	10(100.0)	No	9(100.0)

Figure 13: Malaria symptoms presented - Part 2

Symptoms	Time of observation	Control group (%)		Study group (%)	
Vomiting	Baseline	Yes	2(20.0)	Yes	1(10.0)
		No	8(80.0)	No	9(90.0)
	After T2			No	10(100.0)
	After T3			Yes	1(10.0)

				No	9(90.0)
	After T4			No	10(100.0)
	Final	No	10(100.0)	No	9(100.0)
Diarrhoea	Baseline	No	10(100.0)	Yes	3(30.0)
				No	7(70.0)
	After T2			No	10(100.0)
	After T3			No	10(100.0)
	After T4			No	10(100.0)
	Final	No	10(100.0)	No	9(100.0)
Muscle pain	Baseline	Yes	6(60.0)	Yes	9(90.0)
		No	4(40.0)	No	1(10.0)
	After T2			Yes	4(40.0)
				No	6(60.0)
	After T3			Yes	1(10.0)
				No	9(90.0)
	After T4			Yes	-
				No	10(100.0
	Final	No	10(100.0)	No	9(100.0)
Pain severity	Baseline	Mild	1(10.0)	Mild	6(66.7)
		Moderate	3(30.0)	Moderate	2(22.2)
		Severe	2(20.0)	Severe	1(11.1)
	After T2			Mild	3(60.0)
				Moderate	2(40.0)
	After T3			Mild	1(100.0)
	After T4			-	-
	Final	-	-	-	-
Convul-sion	Baseline	Yes	1(10.0)	No	10(100.0)
		No	9(90.0)		
	Final	No	10(100.0)	No	9(100.0)

Figure 14: Malaria symptoms presented - Part 3

Symptoms	**Time of observation**	**Control group (%)**		**Study group (%)**	
Bloody stool	Baseline	Yes	1(10.0)	No	10(100.0)
		No	9(90.0)		
	Final	No	10(100.0)	No	10(100.0)
Fatigue	Baseline	Yes	10(100.0)	Yes	10(100.0)
	After T2			Yes	5(150.0)
				No	5(50.0)
	After T3			Yes	1(10.0)
				No	9(90.0)
	After T4			Yes	1(10.0)
				No	9(90.0)
	Final	Yes	2(20.0)		
		No	8(80.0)	No	9(100.0)
Malaise	Baseline	Yes	10(100.0)	Yes	10(100.0)
	After T2			Yes	7(70.00
				No	3(30.0)

	After T3			Yes	4(40.0)
				No	6(60.0)
	After T4			Yes	3(30.0)
				No	7(70.0)
	Final	Yes	4(40.0)		
		No	6(60.0)	No	9(100.0)
Body aches	Baseline	Yes	10(100.0)	Yes	9(90.0)
				No	1(10.0)
	After T2			Yes	6(60.0)
				No	4(40.0)
	After T3			Yes	2(20.0)
				No	8(80.0)
	After T4			Yes	1(10.0)
				No	9(90.0)
	Final	Yes	2(20.0)		
		No	8(80.0)	No	9(100.0)

Figure 15: Malaria symptoms presented - Part 4

Conclusion

This study is the first to evaluate anti- microbial photodynamic therapy (using riboflavin as sensitizer) with subsequent intravenous laser therapy as a safe and effective treatment option for malaria.

Haematocrit values and white blood cell counts were gathered to evaluate the safety. There were no statistically significant changes of these values during or after the therapy cycle, indicating that the applied treatment protocol is safe and did not cause any adverse effects on blood components.

A Malaria parasite screening showed that 50% of control group patients were still tested Malaria positive at the end of the treatment cycle. Significantly better results were observed in the study group: 88,9% of the patients were tested negative for Malaria at the end of the study.

A questionnaire revealed a quicker reduction in the number and severity of symptoms presented by patients in the study group compared to those in the control group. Symptomatic improvement could be observed in all patients in the study group except for one.

The obvious weakness of the study is the small patient number with only 10 patients in the treatment group. However, the obtained results suggest that the applied protocol is a safe and effective treatment option for malaria. There is a clear need for follow-up studies with larger patient numbers. A corresponding study will be launched by the authors of this article in summer 2019.