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Sequential analysis of erythrocyte aggregation in *P. falciparum* malaria with and without ASAQ therapy by optical signal and image analysis

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The aggregation of erythrocytes is an important mechanism for blood flow through the cardiovascular system. In malaria, this is complicated by infection caused by *P. falciparum* and is further complicated by the severity of parasitemia. Hence analysis of this micro-mechanism is essential to know the changes in blood not only in diseased conditions but also after artemisinin combination therapy (ASAQ) to alleviate suffering. For analysis purposes, aggregation of erythrocytes was determined by LED laser aggregometer, represented in terms of various parameters related to the changes in laser transmitted intensity. Formed aggregates are further analyzed by imaging and image-processing methods. For this study blood samples from young adults (18 – 40 years old) infected with *P. falciparum* (n= 80), without any other serious illness, were performed. These samples were selected based on the severity of parasitemia, and were divided into low (LP), medium1 (MP1), medium 2 (MP2), and high (HP) parasitemia. For three days, the selected individuals were treated with artemisinin-based combination therapy ASAQ (Artesunate 4 mg/kg and amodiaquine 10 mg base/ kg once a day). Healthy subjects (n=20) without any history of the disease were selected as a control group. The results, as obtained by various parameters, show a significant elevation of aggregation of erythrocytes ($P< 0.05$) in *P. falciparum* malaria with the increase of parasitemia level. There was a decrease in the aggregation after treatment on day four tending towards normal. Thus the current study shows the potential beneficial role of ASAQ on erythrocytes aggregation, which may contribute to reducing the harmful effects on various organs in *P. falciparum*-infected blood.

Keywords: Artemisinin combination therapy, LED Laser aggregometer, *P. falciparum* malaria, Parasitemia aggregation parameters

Erythrocyte aggregation is a fundamental characteristic of healthy blood that plays a significant role in the cardiovascular system, especially in the microcirculation[1]. During malaria, RBC aggregation is further governed by adhesion ligands on infected RBC cells, receptor expression on uninfected RBC, coagulation factors, platelet mediated agglutination, and plasma proteins[2,3]. The parasite invasion, internalization, and growth cause a myriad of erythrocytic changes that lead to different microrheological dysfunctions. These are erythrocytes stickiness and rosetting, compromised, if not entirely abolished, red cell deformability, hyper-aggregation, agglutination tendencies, and non-deforming erythrocyte clumping, resulting in low flow and clogging of blood vessels[4,5]. These dysfunctions in *Plasmodium falciparum* (PF) malaria play a significant role in the sequestering of parasitized red blood cells (PRBCs) in internal organs[6-10] trappings of cells in reticuloendothelial system[5], and blocking of cerebral capillaries[8].

The multiple life cycle stages of *Plasmodium* provide a number of targets for antimalarial chemotherapy. However, all drugs in clinical use for the treatment of malaria act primarily against the intraerythrocytic development of *Plasmodium* parasites. The most important drugs currently in use, for the treatment of clinical *P. falciparum* malaria are focused either on the food vacuole of ring-stage and trophozoites of blood-stage malaria or on enzymes in the trophozoite folic acid biosynthesis pathway[11]. Drugs that have been used clinically that have one of these two modes of action include chloroquine, amodiaquine, quinine, sulfadoxine-pyrimethamine, artemisinin derivatives (predominantly artemether and artesunate), and lumefantrine. Some of these drugs cause dose-dependent hemolysis of erythrocytes[12]. The changes and recovery in red cell indices, white blood cell, and differential and, platelets counts were compared. The highest hematological recovery

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occurred in the amodiaquine artesunate (AQAS) combination therapy treatment group[13].

The study also showed that treatment type and parasitological cure was associated with hematological recovery. In another study[14] investigation on the effect of anti-malaria drugs on some blood cell lines parameters in adult individuals infected with acute uncomplicated *Plasmodium falciparum* malaria showed a lower count of WBC in post anti-malaria drug treatment in all age groups compared to pre-anti-malaria drug treatment. The varying effects of Chloroquine and Coartem on various hematological parameters in rats are observed[15]. All the above studies provided important information on the hematological changes associated with antimalarial drug treatment but none of them analyzed the changes in erythrocyte aggregation, which play an important role in maintaining the hemorheological properties of blood in the cardio-circulatory system.

For detection of these cellular-level changes, an optical technique based on variation of transmitted light intensity is developed. Earlier image processing applications of this technique, based on shape descriptors, include detection of effect of cigarette smoking on erythrocyte aggregation[16]. In the present study, the optical signal is processed to obtain various aggregation related parameters. The malarial parasitic and recovery changes by ASAQ in erythrocytes aggregates are monitored by various parameters, supported by shape descriptors. This forms the objective of the present work and is carried out by optical systems.

Materials and Methods

Experimental technique

Computer-based LED laser aggregometer

A computer based LED laser aggregometer was constructed and the system consisted of a 5 mW LED laser source of wavelength 650 nm, continuous output, and a round dot beam of diameter 1.0 mm. The specimen chamber consists of optically flat glass plates with internal dimensions 8.0 × 1.6 × 70 mm to allow interaction between the cells and sedimentation of the formed aggregates[17,18]. The variation in the transmitted intensity (TI) variation is measured by the signal emitted from the observation volume (OV) center of the chamber and was detected by the photodiode-amplifier assembly (BPW34). This signal was further amplified and digitized by a NI USB-6000 ADC interface and processed by the computer.

A well-mixed erythrocytes suspension of hematocrit 5% in plasma was placed in the chamber up to a height of 60 mm. Due to the movement of the formed aggregates in observation volume (OV) during the sedimentation process, the TI signal is super-posed with fluctuations, which are characteristic of the size of aggregates. The signal was recorded for 5 min. By analysis of the TI signal, the following parameters of were obtained:

(i) Net induced change (NIC): It indicates the net change in the transmitted intensity (TI) given by the expression TI max – TI min, where TI min and TI max are the variation in intensity at the beginning of the record and the ending, respectively.

(ii) Total number of fluctuations (TNF): It is the number of fluctuations observed in the TI during the movement of formed aggregates.

(iii) Aggregation sedimentation time (AST): This parameter indicates the time duration for the formed aggregate to sediment through the observed volume (OV) and is determined by, OV=T2 − T1, where T1 and T2 are time intercepts on the left and right sides of the curve, respectively.

(iv) Aggregate amplitude height (AAH): It is calculated from the baseline of each amplitude and indicates the size of aggregate.

(v) Aggregates open area (AOA): This parameter indicates the change in the compactness of the aggregates and is determined from measured the open area.

Sample preparation

Blood samples

After obtaining written consent the patients or their attendant relatives, blood samples were obtained from eighty young adult patients (Forty of 18 to 29 years and Forty of 30 to 40 years). The analysis was carried out at one of three Malaria Sentinel Sites of the Democratic Republic of Congo (DRC) in North-Kivu Province. Samplings were done after overnight fasting between 8 am and 10am in a quiet environment at an average ambient temperature (25°C). Blood withdrawal was made after a ten-minute resting period and in a seated position. At the time of this study, participating patients shouldn't have received any other anti-malarial treatment. The Health National Ethical Committee of the Ministry of Public Health, Democratic Republic of Congo, approved this investigation.

Individuals below eighteen years and above forty years, pregnant ladies, people with sicknesses associated with red blood cells, those who had blood

transfusions shortly once admitted, and people who have had previous antimalarial treatment at intervals of 24 hours of admission were excluded from the study. Samples were taken before treatment and on the fourth day. Patients participating in the study were subjected to a fixed-dose combination tablet of Artesunate amodiaquine (ASAQ) for three days. It was given once daily orally at one strength of combination (AS: 100 mg/AQ: 270 mg) formulated for all selected groups. A daily visit was organized to assure all subjects are respecting the given prescription. Age and sex-matched twenty healthy subjects with no history of diseases were designated as a control group.

Parasitemia classification — Based on the parasitized RBC count, ranging from 1, 2-3, 4-5, and more than 5, these samples are classified as low (LP), medium 1(MP1), medium 2(MP2), and high (HP) parasite density, respectively.

Aggregation measurement — Fresh venipuncture blood samples were collected in the test tubes containing citrate dextrose phosphate as an anticoagulant.

Each blood sample was centrifuged (3000 rpm for 20 min at room temperature), the supernatant plasma was separated, and the buffy coat on the top of the cells was separated and discarded. The suspensions of 5% hematocrit in the plasma was prepared for aggregation measurement. These measurements were carried out in a room at 25±1°C.

Aggregation detection and analysis

Aggregates detection

Two methods were adopted to detect aggregates:

(i) *Method 1* — The prepared specimen chamber was filled with well-mixed erythrocytes suspension and placed between the source of the laser beam and the detector. The erythrocytes aggregate formed in the chamber, sediment under the gravitational field, and pass through the observed volume. The transmitted intensity varies depending on the size of the aggregates present in the path of the laser light beam. Data were recorded at a sampling rate of 100 data per second using LABVIEW software sequentially for 5 min, the time estimated to give critical information on the formation of aggregates. The transmitted intensity was digitized and stored for further analysis.

(ii) *Method 2* — It consists of a simple slide test and image analysis to reveal the state of erythrocyte aggregation. Blood was drawn into a test tube containing citrate dextrose phosphate. A drop of blood was placed on a slide that was held for 2 to 3 seconds at an angle of 45° so that the blood could run down by gravity, leaving an excellent film. The slides were then dried within 10 min at room temperature placed in a completely horizontal position and using the GIEMSA slide Steiner for further analysis by the Olympus BX 63 image analysis system. The fields of view were chosen systematically to sample different regions on the slide. Each image was processed separately with the help of MATLAB software.

Aggregates analysis

The acquired data from method 1 was analyzed to extract the following hemorheological parameters: Net induced change (NIC), the total number of fluctuations (TNF), aggregation sedimentation time (AST), aggregate amplitude height (AAH) and aggregates open area (AOA). The images acquired from method two were processed by a simple automated method to analyze the red blood cell aggregation by comparing the open area present in the images of aggregates found in both malaria and standard samples. From the measured number of pixels along the open area and multiplied by the pixel area, the open area in the aggregate image was determined. Figure 1 shows the flow chart for the measurement of aggregates open area.

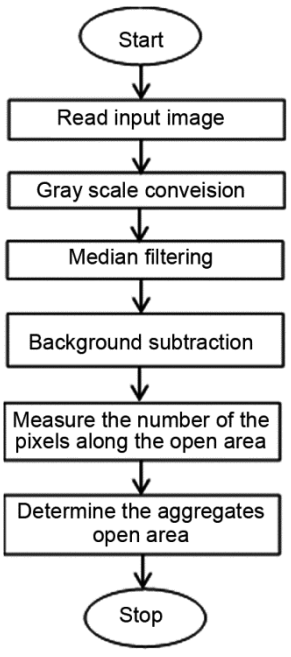

Fig. 1 — Flow chart for measurement of aggregates open area

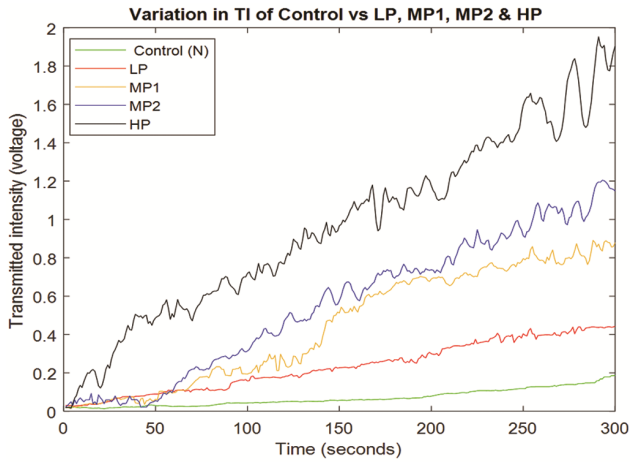

Fig. 2 — Variation in transmitted intensity obtained during aggregation process of normal subjects and malarial parasitaemia of various categories on day 1

Statistical evaluation

Statistics analysis has been carried out by using the SPSS software package (IBM SPSS information version 23) and expressed as mean ± standard deviation. Student's *t*-test and analysis of variance (ANOVA) were utilized in evaluating values between and amongst the mean of groups, respectively. P-values much less than 0.05 (P <0.05) at a 95% confidence limit was considered significant, while P-values larger than 0.05 (P >0.05) at a 95% confidence restriction were considered as nonsignificant.

Results

Figure 2 shows the variation of transmitted intensity (TI) of erythrocyte suspension of healthy, low, medium1, medium 2, and high parasitemia samples. There is a gradual increase of TI with large fluctuations in amplitude as the process time increase and with the increase in parasitemia level compared to normal. The amplitude of the fluctuations varies, indicating the change in aggregate size that crossed the observed volume due to gravity. Initially, due to a well-mixed sample having the same hematocrit in observed volume, the value of minimum TI is almost the same for healthy and all parasitemia categories. As time increases, TI's value increases considerably with the level of parasitemia compared to control subjects throughout the sedimentation process. This phenomenon is due to faster sedimentation of large aggregates formed in parasitized samples than in control.

More fluctuations are present in parasitized samples from the beginning of the process compared to normal. Table 1 shows a statistically high

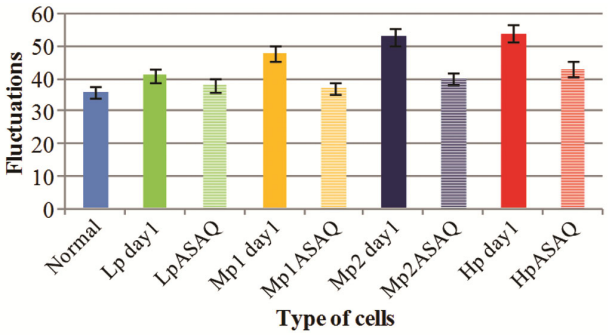

Fig. 3 — Variation in total number of fluctuations (TNF) of Normal, Low parasitemia, Medium parasitemia 1, Medium parasitemia 2 and High parasitemia on day 1 before treatment and on day 4 after treatment

Table 1 — Comparison of mean NIC of normal subjects and malarial parasitaemia of various categories on day 1 before treatment and on day 4 after treatment

Samples (n=20)	Net Induced Change (NIC) Day 1 before treatment	Net Induced Change (NIC) Day 4 after treatment
Normal	0.17±0.012	0.17±0.012
LP	0.44±0.02**	0.177±0.017
MP1	0.87±0.021**	0.2±0.017*
MP2	1.18±0.06**	0.29±0.01**
HP	1.93±0.034**	0.32±0.02**

P values: * <0.05, ** <0.001
NIC – net induced change

significant difference in mean NIC with the increase of parasitemia level. (P <0.001) and a decrease in trend on day four after treatment. The above results suggest that with the increase of parasitemia, the *P. falciparum* malaria parasite, after its invasion, produces some changes in the erythrocyte. The decrease in NIC on day four tending towards normal suggests that the given treatment contributed to the recovery of RBC at the early stage. The World Health Organization (WHO) protocol for evaluating an antimalarial drug or drug combination now includes hematological recovery as an efficacy endpoint[19].

Figure 3 shows the variation in the total number of fluctuations (TNF) of Normal, Low, Medium 1, Medium 2, and High parasitemia before treatment and after treatment. The appearance of fluctuations in the signal indicates the sedimentation of the formed aggregates. The number of fluctuations in High parasitemia is the maximum. The results show a decrease in TNF tending towards regular after treatment on day 4. The mean total number of fluctuations (MTNF) of various parasitemia levels and healthy is further compared statistically (Table 2).

Table 2 — Comparison of mean TNF of normal, Lp, Mp1, Mp2 and Hp on day 1 before treatment and on day 4 after treatment

Samples (n=20)	Total Number of Fluctuations (TNF) Day 1 before treatment	Total Number of Fluctuations (TNF) Day 4 after treatment
Normal	7.20±5.1	7.2±5.4
LP	8.60±3.9	7.6±5.4
MP 1	9.80±2.9	7.4±4.9
MP 2	10.20±4.2	8±2
HP	10.80±2.2	8.6±1.14

P values: * <0.05, ** <0.001
TNF -total number of fluctuations

Table 3 — Comparison of mean AST of normal, Lp, Mp1, Mp2 and Hp on day 1 before treatment and on day 4 after treatment

Samples (n=20)	AST (in seconds) Day 1 before treatment	AST (in seconds) Day 4 after treatment
Normal	1.4±0.31	1.4±0.31
LP	1.59±0.44	1.42±0.32
MP 1	2.43±0.75*	1.4±0.29
MP 2	1.94±0.6*	1.43±0.33
HP	2.53±0.65*	2.32±0.58*

P values: * <0.05, ** <0.001
AST – aggregation sedimentation time

Table 4 — Comparison in AAH of normal, Lp, Mp1, Mp2 and Hp on day 1 before treatment and on day 4 after treatment

Samples (n=20)	Aggregate amplitude height (in Volts peak) Day 1 before treatment	Aggregate amplitude height (in Volts peak) Day 4 after treatment
Normal	0.0011±0.0009 (N = 36)	0.0011±0.0009 (N = 36)
LP	0.0024±0.0003* (N = 41)	0.0013±0.0002 (N = 39)
MP 1	0.012±0.002** (N = 48)	0.0021±0.00023* (N = 37)
MP 2	0.027±0.0032** (N = 53)	0.0016±0.0003* (N = 40)
HP	0.051±0.006** (N = 54)	0.0081±0.0012* (N = 43)

P values: * <0.05, ** <0.001
AAH -aggregate amplitude height

Table 5 — Comparison of AOA of normal, Lp, Mp1, Mp2 and Hp on day 1 before treatment and on day 4 after treatment

Samples (n=20)	Aggregates Open Area (pixels area in μm ²) Day 1 before treatment	Aggregates Open Area (pixels area in μm ²) Day 4 after treatment
Normal	5227.95±282.3	5227.95±282.3
LP	5398.54±136.15*	5235.67±108.58
MP1	5469.44±68.87*	5245.46±117.9
MP2	5534.70±117.9*	5234.24±170.8
HP	5593.59±56.76*	5241.69±213.5

P values: * <0.05, ** <0.001
AOA - aggregates open area

An increasing trend of mean TNF without a statistically significant difference was observed. This process indicates the increasing hyper aggregation tendency of erythrocytes in patients with an increase in parasitemia level.

On day 4 the MTNF of all categories decreased with no significant difference compared to that of the control group. This could be attributed to the interaction of parasitized cells and the used antimalarial drug.

Table 3 shows the comparison in the aggregate sedimentation time (AST) of healthy and malaria samples on day 1 before treatment and day 4 after treatment. The AST is a minimum for healthy and maximum for HP malaria. The increase of PRBC in the sample reduces the overall deformability of the erythrocytes, while the data of AST shows no significant difference for LP, Mp1, and Mp2, a significant difference for the HP (P <0.05) category compared to that of healthy is observed.

There is a decreasing trend of the AST on day four after treatment without a significant change of Lp, Mp1, and Mp2 compared to the control group. This decreasing trend suggests that the administration of the anti-malarial drug improves the overall sedimentation of the erythrocytes.

There was an increase in aggregate amplitude height (AAH) with the increase inparasitemia level. The AAH is minimal in healthy and maximum in Hp. The mean AAH of various parasitemia levels and normal is further compared statistically (Table 4). While the data of AAH shows a significant difference for LP (P <0.05), a highly significant difference for the Mp1, Mp2, and HP (P <0.001) categories is observed compared to that of healthy. The results show that the treatment induced a decrease in aggregate amplitude height (AAH) tending towards normal. There was an increase in the aggregate open area (AOA) with an increase inparasitemia levels. The above results indicate that aggregates are compacted, leading to a decrease in their occupied area. The erythrocytes tend to adhere to each other forming clumps with minimum deformation, as seen in aggregates images (Fig. 4). The mean AOA of various parasitemia levels and normal on day one before treatment and on day 4 after treatment is further compared statistically (Table 5). The data shows a significant difference in the Lp, Mp1, Mp2, and HP (P <0.05) categories compared to that of healthy. The clearance of malaria parasites induced the decompression of the aggregates in the form of rouleau as seen on day four. This suggests that the

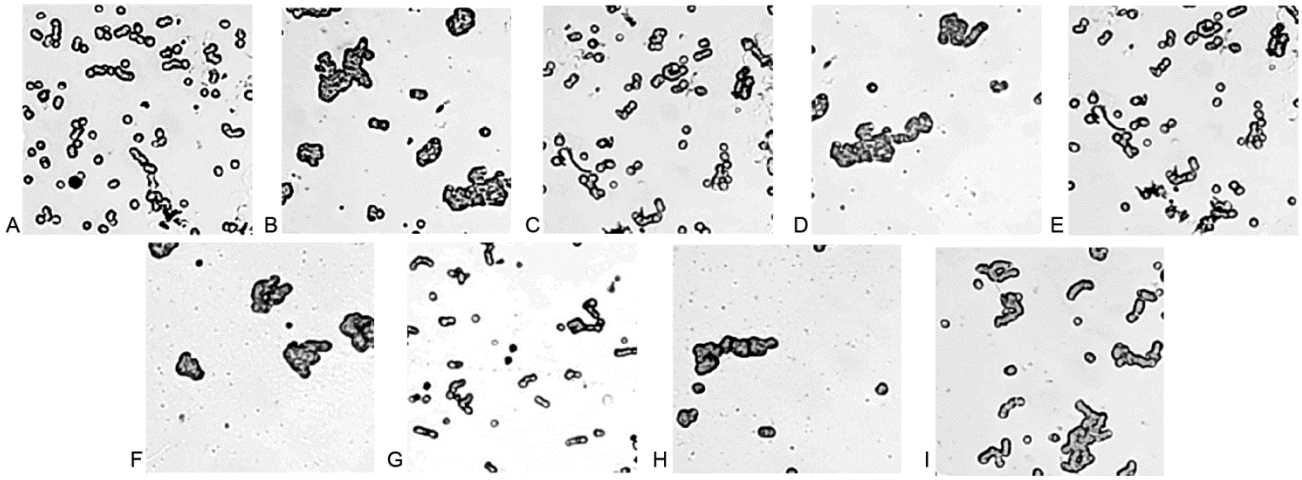

Fig. 4 — Images of aggregation process of normal (A) Lp day 1; (B) Lp day 4; (C) Mp1day 1; (D) Mp1day 4; (E) Mp2 day 1; (F) Mp2 day 4; (G) Hp day 1; (H); and Hp day 4 (I)

administration of the ASAQ anti-malarial drug reduces the *P. falciparum* inflammatory activity early leading to the decrease of the erythrocytes sedimentation process.

Discussion

RBC aggregation is a physiological event required for maintaining normal homeostasis of the body. RBCs have the tendency to interact with each other within a blood vessel during flow to form lower-level aggregates containing 2 or 3 cells, but these aggregates are very weak and disintegrate when passing through high shear stress. Under pathophysiological conditions, favoring RBC aggregation, results in the formation of big RBC aggregates responsible for vesicular blockage and tissue damage[20]. The intensity of aggregation has been used as an index of the severity of pathological conditions such as cardiovascular diseases, diabetes mellitus, occlusive diseases, and microangiopathy[21].

In the present study, we have observed the changes in erythrocyte aggregation due to *P. falciparum* malaria under increasing disease severity conditions and in post-treatment at the early stage (on day 4). Recently, the World Health Organization suggested that for therapeutic efficacy studies for *P. falciparum* when treated with ASAQ, the patient is closely monitored during the first 3 days to ensure that appropriate rescue treatment is given if signs of severe malaria develop[22]. Such monitoring is crucial for early treatment failure (ETF) detection.

The malarial samples are divided into four severity categories (LP, MP1, MP2, and HP) based on the PRBC count in blood smear slides. Such a classification is appropriate as a higher parasitemia level corresponds to greater severity of the infection and acute phase of malaria[23].

Results from previous studies have shown significant changes in erythrocytes aggregation during malaria infection[2,3]. This phenomenon was confirmed in this study, as results revealed a statistically significant increase in aggregation parameters in malaria-infected subjects compared to non-malaria-infected subjects.

The present results clearly show the change in RBC aggregation in malaria concerning the different levels of parasitemia. This agrees with early studies. The results indicate that with increasing parasitemia, the erythrocytes tend to adhere to each other forming compacted clumps as seen in aggregates images (Fig. 4). As the membrane properties are subjected to change in parasitized samples, the formed aggregates show compactness in Mp1, Mp2, and Hp categories compared to the control aggregate. This clumping may be attributed to the enhanced cellular adhesion in malaria on account of cytoadhesion, platelet glycoprotein-mediated auto-agglutination[24,25], and other cellular aspects[26]. A study on adhesive phenotypes involved in auto-agglutination and rosetting in malaria through electron micrographs[25] confirmed that the auto-agglutinates or clumps were also composed of infected erythrocytes and platelets. These platelets acted as bridges between the parasitized erythrocytes.

Results from this study also showed that aggregation parameters, notably the NIC and AOA, are significantly altered in all levels of parasitemia

compared to that of healthy, thus further supporting the early enhanced aggregation in *P. falciparum* malaria. As the changes in hemorheological parameters for a given disease are considered to be indicators for the disease severity[27], these may be chosen for the description of *P. falciparum* malaria's acuteness to supplement the parasitemia-based classification. The AST was maximum in Hp and minimum in healthy samples. This parameter is the reciprocal of the erythrocyte deformability. An increasing trend in AST from Lp to Hp shows that the overall deformability of the cells decreases with the increase of parasitemia. This finding is in agreement with our previous study[28].

Furthermore, our study highlights another facet of the potential beneficial role of ASAQ on erythrocytes aggregation during malaria. This study revealed that ASAQ used to treat malaria patients significantly improved the aggregation parameters at the early stage. Interestingly, the control done on day four after treatment showed that lower-order aggregates were mostly present in ASAQ-treated RBCs and mimics physiological conditions. Hence, ASAQ may help break/inhibit the formation of bigger RBC aggregates leading to physiological RBC aggregation.

The sticky forces between infected and uninfected RBCs slow down the micro-circulatory flow[29], but the molecular mechanism and master player are not known. Several factors, such as changes in shear stress, protein-protein interactions, and erythrocyte membrane charge alteration, regulate the RBC aggregate formation[3,30]. During malaria, oxidative insult makes RBC membrane sticky to bind infected RBC cells expressing PfEMP-1 to form rosettes and aggregates[5,31]. Both knobs and PfEMP1 are thought to contribute to IRBC sequestration, giving rise to enhanced disease severity through small vessel occlusion, tissue ischemia, and eventual organ failure.

The anti-malaria activity of artemisinin and its derivatives[32,] is exceptionally rapid, and most patients show clinical improvement within 1-3 days after treatment[14,33,34]. The World Health Organization indicated in a recent report that artemisinin-based combination therapies are still effective against malaria parasites and that their use is still recommended[35].

Conclusion

This present work supports the significant elevation of aggregation of erythrocytes in *P. falciparum* malaria with the increase of parasitemia level. Results obtained showed that aggregation parameters, notably the NIC and AOA, were significantly altered in all levels of parasitemia when we compared test subjects with controls. We also obtained an increasing trend in AST from Lp to Hp when the test and control subjects were compared. In contrast to morphological analysis of erythrocytes by micro-photograph techniques[36] the present methods are applied to determine the aggregation characteristics of erythrocytes by imaging and image analysis.

In the current study, we highlighted the potential beneficial role of ASAQ on erythrocytes aggregation in *P. falciparum*-infected blood. ASAQ treatment improved the aggregation parameters significantly at the early stage and gave new insight into its contribution to erythrocytes recovery. Hence, our results suggest that ASAQ could help in breaking/inhibiting the formation of bigger RBC aggregates leading to physiological RBC aggregation.

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Conflict of interest

All authors declare no conflict of interest.

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