

In vitro growth of *Plasmodium falciparum* field isolates in homozygous and heterozygous sickle cells

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Abstract:

Background: *Plasmodium falciparum* malaria is a major cause of morbidity and mortality throughout human history. The best-characterized human genetic polymorphism associated with malaria results in sickle haemoglobin (HbS). The high prevalence of HbS in sub-Saharan Africa and some other tropical areas is almost certainly due to the protection against malaria afforded to heterozygotes, many *in vivo* and *in vitro* studies were done to explain the protection mechanisms. The present study investigated the possibility of *in vitro* growth of *P. falciparum* field isolates in erythrocytes from different two sickle cell trait two sickle cell disease and four Sudanese donors (normal haemoglobin).

Subjects and Methods: This is *in vitro* experimental study carried out in four study participants from three different tribes Bargo, Messeria and Shaigia two are homozygous and two are heterozygous sickle cell anemia which confirmed by positive electrophoretic band of HbSS and HbAS respectively, Three *P. falciparum* field isolates were co-cultured in normal and sickle cells. Parasite cultures were done in RPMI1640 supplemented with Albumax.

Results: *P. falciparum* completed asexual blood stage after 48 hours incubation homozygous sickle cells, sickle cells enhance parasitaemia by 12 fold compared with initial parasitaemia after 48 hours incubation, while parasites failed to complete the asexual blood stage in heterozygous sickle cells and remained extracellular with abnormal morphology.

Conclusions: The present study showed that the *P. falciparum* can grow in homozygous sickle red blood cell but failed to grow in heterozygous sickle red blood cell.

Key words: in vitro, *Plasmodium falciparum*, homozygous and heterozygous sickle cells

Introduction:

Plasmodium falciparum malaria is a major cause of morbidity and mortality throughout human history. As a result, malaria has exerted extraordinary evolutionary pressure on the human genome and appears to have selected for multiple genetic polymorphisms that provide protection against severe disease^{1,2}. The best-characterized human genetic polymorphism associated with malaria results in sickle haemoglobin (HbS). The high prevalence of HbS in sub-Saharan Africa and some other tropical areas is almost certainly due to the protection against malaria afforded to heterozygotes^{1,3,4}. Since the protective effect of sickle cell trait on malaria was first described over 60 years ago^{5,6}. Sickle haemoglobin (HbS) is a structural variant of normal adult haemoglobin. Adult haemoglobin (HbAA) is made up of two alpha and two beta globin chains. HbS is the result of a single point mutation (Glu→Val) on the sixth codon of the beta globins gene⁷. Homozygotes for haemoglobin S (HbSS) with two affected beta chains develop sickle cell disease, in which polymerized haemoglobin causes red blood cells to sickle and occlude blood vessels, Vaso-occlusion affects many organs and tissues, and

results in high morbidity and mortality. Heterozygotes for sickle haemoglobin (HbAS) have sickle cell trait and are generally asymptomatic⁸.

About 50 years ago, it was noticed that the incidence of sickle cell trait with HbAS erythrocytes was higher in regions where malaria was prevalent than elsewhere⁹. That observation has been repeatedly confirmed over the years and it is now widely accepted that sickle cell trait confers partial protection against severe falciparum malaria¹⁰. In epidemiological sense, the protection provided by sickle cell trait against death from malaria somehow compensates for the health devastation inflicted by sickle cell disease¹¹. Unfortunately, individuals with sickle cell anemia (HbSS) are not protected from falciparum malaria, perhaps because of their pre-existing poor health¹². The specific mechanisms of protection were studied in a group of them: some are in vivo and others affect parasite in vitro and apply this theory as occurs in vivo. Arif collected possible causes of protection done in the world which are; Red cells of patients of sickle cell trait, when infected with the *P. falciparum* parasite, deform, most probably because the parasite reduces the oxygen tension within the erythrocytes to very low levels as it carries out its metabolism. Deformed sickle trait red cell when passes through splenic sinusoids sequestered out by the phagocytes¹³. Similar result was also obtained by Kodjo et al where in vitro experiment showed increased phagocytosis of ring parasitized RBCs in Hb AS individual by mononuclear phagocytic system of spleen whereas trophozoite-parasitized normal RBC and RBC in HbAS were phagocytosed at equal pace. They concluded that ring parasitized RBC in sickle cell trait patients was predominantly complement mediated and very similar to phagocytosis of senescent or damaged normal RBCs. It was also concluded that the phagocytosis of ring parasitized red cells by monocytes have advantages in various ways, firstly it reduces parasite growth and density, secondly phagocytosed ring parasitized red cells digest rapidly by monocytes and process is

repeated without loss of function whereas more mature form of parasite actively phagocytosed and severely affect the function of monocyte¹⁴. In another study in vitro experiment showed that sickle trait red cells infected with *P. falciparum* malaria sickled much more readily than uninfected cells under low oxygen tension¹⁵. Since sickle cells are removed from the circulation and destroyed in the reticuloendothelial system, it reduces the parasite burden in people with sickle trait. These people are more likely to survive acute malarial infections. It was further highlighted in another study that malaria parasites could be killed directly in sickle red cells. When parasites were cultured in sickle trait red cells and incubated at low oxygen tension, they died¹⁶. Ultra structural studies showed extensive vacuole formation in *P. falciparum* parasites in sickle trait red cells when incubated at low oxygen tension, suggest that there is some metabolic damage to the parasites¹⁷. Other investigations show that there is oxygen radical formation in sickle trait erythrocytes and it retards growth and even kills *P. falciparum* parasites. There is more production of super oxides (O^{2-}) and hydrogen peroxide (H_2O_2) in sickle trait red cell than the normal erythrocyte¹⁸. There is formation of membrane associated hemin in homozygous hemoglobin S red cell which can oxidize membrane lipid and protein. However sickle trait red cell produce little hemin. But infected sickle trait red cells owing to formation of sickle polymer due to low oxygen tension and due to parasite metabolism produce enough hemin To damage the parasites^{19,20}. Immune system also plays an important role in attack of *P falciparum*. Maternal antibodies passed to fetus provide protection from malaria for first few months of life. There after toddler's immune system provides protection. Many epidemiological studies conducted in endemic areas show that antibody titer of *P. falciparum* are lower in children in sickle cell trait than in normal children. It is speculated low level of titer might reflect a low parasite burden in children with sickle trait due to clearance of infected red cells. In contrast children

suffering with sickle cell disease have high fatality rate when infected with *P. falciparum* parasites^{21,22, 23}.

Subjects and Methods:

This is in vitro experimental study carried out in four study participant from three different tribes Bargo, Messeria and Shaigia two are homozygous and two are heterozygous sickle cell anemia which were confirmed by positive lectrophoretic band of HbSS and HbAS respectively, two heterozygous participant are adult females and have no history of transfusion from birth until the sample drawn, two homozygous participant are children and have no history of transfusion sixth month before sample drawn. The study was conducted in Khartoum state in the duration period from August to November 2013. The medical history was taken carefully from each participant in order to collect information about his general health, age, gender, tribe, history of transfusion. Participants were excluded from the study in case of history of transfusion less than four months, sickle cell crises, participant in ICU unit, and who are not consented. For normal participant Hb AA positive blood film or antibody for *P.falciparum* were excluded from study.

Ethics statement

The study received approval from the Ethics review committee, Alzaeim Alazhari University.

Preparation of erythrocytes for culture

All samples were collected in Citrate Phosphate Dextrose Adenine which is used routinely in blood bank to make the cells viable until the culture achieved, the percent of anticoagulant is that range used in blood bank 63 to 450 ml we add 0.7 ml to 5ml blood²⁴. Washing of erythrocytes three times were done by

sterile phosphate buffer saline (sterilization done by filtration through 0.2 micro meter pore size) instead of incomplete malaria culture medium washing were done by centrifuged cells 3000rp/min for 10 minutes leukocytes are removed and red cells pellets were used in culture .(preparation of culture medium for parasite growth) available commercial liquid RPMI 1640 medium with stable L Glutamine (Biowest) were used in the culture, preparation of working medium is achieved by adding 10g/L Albumax 1 (Gibco) and 0.5ml/L Gentamycin (Sandoz) (from 50 mg/mL stock)²⁵.

Parasite culture

Parasites were grown essentially by standard in vitro method²⁶. Unless stated otherwise, the cultures were contained in 50 ml culture flasks ²⁷. As in our experiment the continuous culture is difficult to achieved in many culture from patient isolated parasite, homozygous Hb SS erythrocytes directly diluted with parasite from patient isolate to get appropriate parasitaemia and we compare the growth with normal Hb AA erythrocytes, in Hb AS erythrocytes adding of AS erythrocyte after 24 hours after growth in Hb AA erythrocytes. All culture flask incubated in candle jar, flask were labeled with date of culture type of cells homozygous heterozygous or normal before incubation. A burning candle was placed in glass desiccators containing the parasite cultures. The desiccators was covered tightly with the cover, after which the candle fire extinguished by itself within 5 min. all process were done aseptically in laminar hood and using of electrical pipette with disposable plastic pipette ranging from 1ml to 10 ml to avoid the contamination of culture.

Assessment of growth

Assessment of growth was made after taking of few milliliter of cells by sterile pipette put it in pendorf tube centrifuged remove the supernatant (media) and made thin film, stained by

Giemsa stain by standard methods described here, air-dry the thin film, fix the film in methanol for about 30s, prepare a fresh 5 to 10% Giemsa solution in phosphate buffer (pH 7.1), put the slide in a staining jar or on a staining rack and pour the Giemsa solution on the slide, leave it to stain for 20 min., rinse it carefully and thoroughly under running tap water, leave the slide in an upright position to dry and observe the film with immersion oil and objective at 100 \times ., evaluate and count parasitaemia (An area of stained thin blood film where the erythrocytes were evenly distributed, was observed using 100 X objective, under oil immersion). Approximately 100 erythrocytes in this area were counted. Without moving the slide, the number of infected erythrocytes amongst the 100 erythrocytes was also counted. The slide was moved randomly to adjacent fields and counting was continued as mentioned above. An equivalent of 1,000 erythrocytes was counted. The counting was repeated twice for a total examination of three different parts of the slide, i.e., 3 areas 1000 cells. The mean number of infected RBCs per 1,000 RBCs was taken by dividing the infected RBCs by 3.

Calculation:

A = number of infected erythrocytes/1,000 erythrocytes
Percent infected erythrocytes (% parasitaemia) = $A/10^{25}$

Results:

Growth and multiplication of *P. falciparum* in HbSS erythrocytes: two *P. falciparum* parasites from different patients were completed the asexual life cycle in two HbSS erythrocytes culture within 48 hrs in candle jar, and the number of merozoites in mature schizont ranged from 5 to 10 merozoite appeared after 24hrs incubation Figure (1,2), parasitaemia after 48 hrs increased 12 fold in comparison to initial parasitaemia 0.5% (after 48 6% parasitaemia were

achieved see Chart (1)) group of cells become sickled after 48hrs show in Figure (3,4). The same parasitaemia of culture in Hb AA erythrocytes culture were achieved. Our results agreed with previous study of Orjih²⁸. Growth and multiplication of *P. falciparum* in HbAS erythrocytes; One *P. falciparum* parasite fail to complete the asexual life cycle in two Hb AS erythrocytes and the predominant parasite remains as extracellular with abnormal morphology after 24 hrs incubation Figure (5) after 48 hrs parasite still remain as extracellular and many cells become sickled Figure (6) no increase in number see Chart (2). This study disagreed with the same study done by Orjih²⁸.

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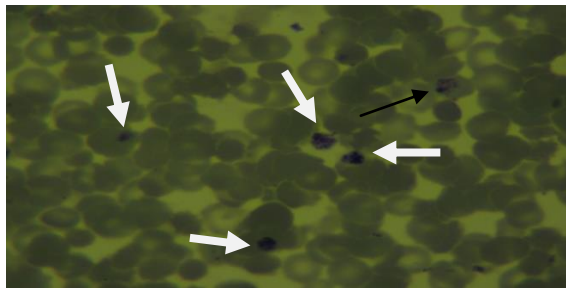


Figure (1) mature schizont's in HbSS erythrocyte after 24 hours incubation

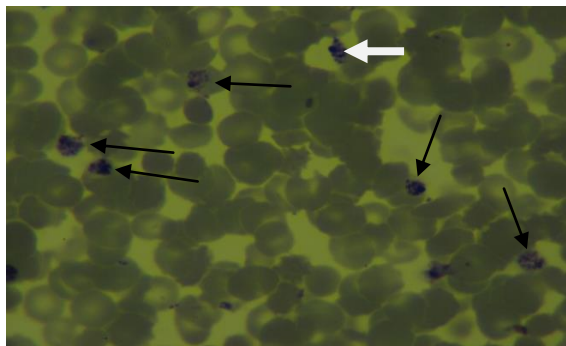


Figure (2) mature schizont's in HbSS erythrocyte after 24 hours incubation from another sample.

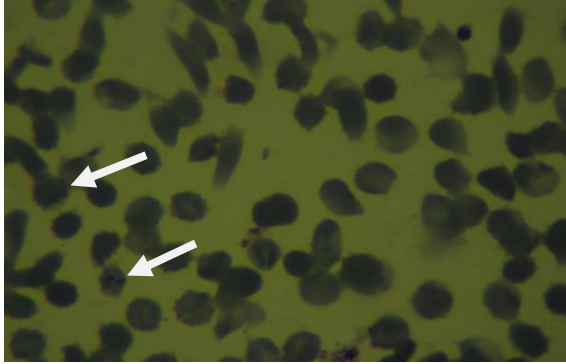


Figure (3) Ring stage in HbSS erythrocyte after 48 hrs and sickle cells.

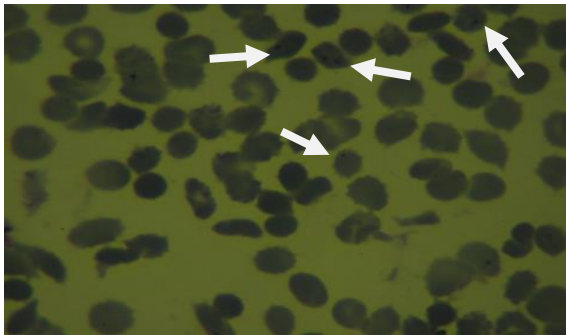


Figure (4) Ring stage in HbSS erythrocyte after 48 hrs and sickle cells from another sample.

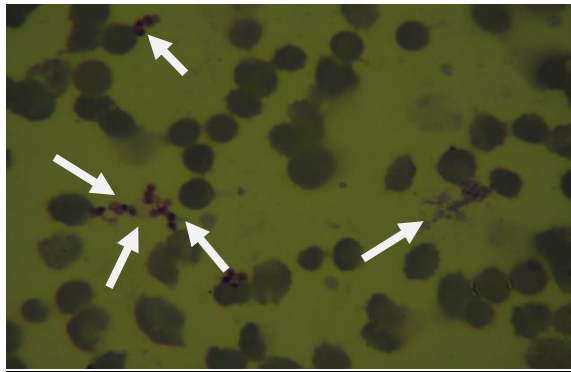


Figure (5) extracellular parasite with abnormal morphology after 24hrs incubation in HbAS erythrocyte.

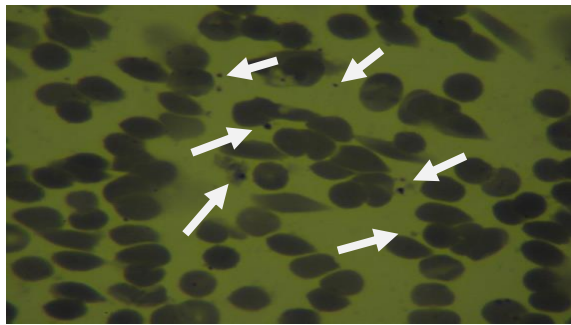


Figure (6) extracellular parasite with abnormal morphology after 48hrs incubation in HbAS erythrocyte.

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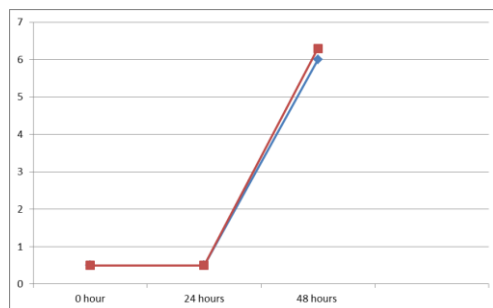
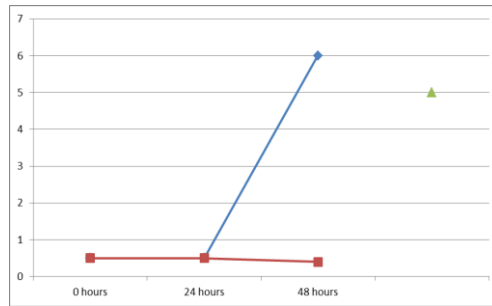


Chart (1) growth rate of homozygous sickle cells (blue line) normal pink line.



Charts (2) growth rate of heterozygous sickle cells (pink line) normal pink blue.

Discussion:

This study is based on in vitro experimental study carried out during period August - November 2013 in Khartoum State to study the ability of *P. falciparum* to grow in culture containing homozygous and heterozygous sickle cells erythrocytes. Two *P. falciparum* isolates from two different patients in Khartoum teaching hospital completed asexual stages in two homozygous sickle cells erythrocytes from different patients from Messaria and Bargo tribes. Our result disagreed with above study done by Orjih²⁸ and explain theory that when children with sickle cell disease (SCD) have a high fatality rate when infected by *P. falciparum*. Failure of growth of another different one parasite which culture in two different heterozygous sickle cells erythrocytes from Shaigia it make benefit for heterozygous to survive which are occurs in our study participant two are adult females, there are many factors explain our results which occurs in heterozygous sickle cells, important and major factors is specific intra-erythrocytic conditions of HbAS red blood cells, such as low intracellular potassium²⁹. High concentrations of haemoglobin³⁰ or osmotic shrinkage of the red blood cell ³¹ cause an inhospitable environment for parasites. Recent data provide support for the intriguing possibility that human micro RNAs translocated into parasite mRNA reduce intra-erythrocytic

growth. This study found two human micro RNAs that were highly enriched in erythrocytes with HbAS, and these micro RNAs inhibited translation of specific parasite mRNA transcripts negatively impacting parasite growth in vitro³³.

Conclusions:

The present study showed that the *P. falciparum* can grow in homozygous sickle red blood cell but failed to grow in heterozygous sickle red blood cell.

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