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PREPARATION AND *IN VITRO* CHARACTERIZATION OF RESEALED ERYTHROCYTES CONTAINING TMR-DEXTRAN FOR DETERMINATION OF HEMOGLOBIN UPTAKE AND TRANSFER BY THE MALARIA PARASITE

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Keywords:

Resealed Erythrocytes, Tmr-Dextran, Hemoglobin, Malaria Parasite, Hypotonic Dilution

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ABSTRACT: The hemoglobin uptake and transport by the malaria parasite, *Plasmodium falciparum* are preferable targets for antimalarial drug development. There is a need for alternative approaches to investigate the endocytic process of live and intact cells under non-disruptive conditions as previous findings were mostly based on morphological analysis of thin-section electron micrographs. In the present study, resealed erythrocytes containing TMR-dextran, the host cell cytoplasm marker, were prepared and characterized. Fresh erythrocytes were collected in EDTA anticoagulant vacutainer tubes, which proved to maintain a normal biconcave disk shape of the cells. Using a modified hypotonic dilution method, washed erythrocytes were lysed in 3 volumes of hemolysis buffer, which permitted the retention of 33.56 ± 7.84% of the original hemoglobin content of the cells. Resealed erythrocytes containing TMR-dextran were invaded by the parasites with similar efficiency to unlabeled cells, and able to support the parasite growth and development. Live cell fluorescence imaging of the endocytic process revealed the appearance of TMR-dextran-containing small endocytic vesicles with intense signals at early ring stage. These compartments coalesced in the early trophozoite stage to form a central digestive vacuole. A larger spherical structure within TMR-dextran labelled ring and trophozoite stage parasites was often observed, which is likely to resemble the previously described "Big Gulp". It is therefore concluded that resealed erythrocytes incorporated with TMR-dextran, which were proved their biological applicability, can be a model for the endocytic study of *P. falciparum.*

INTRODUCTION: The blood stage malaria parasite has developed a number of unusual metabolic and cell biological functions that prepare it for its life within the erythrocytes of its host [1-2]. The feeding process of the parasite forms one of the core foundations of malaria disease, which is responsible for 214 million cases and 438 000 deaths globally each year [3].

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Given the significance of the blood stage of infection to disease pathology, there has been intensive interest in understanding the mechanisms by which the parasite ingests the host cell cytoplasm using endocytic structures, and transfers the hemoglobin to the acidic digestive vacuole [4-6] as a route to develop novel strategies that combat the hemoglobin uptake and transfer, hence, block the malaria disease [7].

With the revolution of fluorescent probes, fluorescence imaging has become a useful tool for studying dynamic cellular events in living cells under physiological conditions [8-9]. Fluorescent probes can be applied to measure ion

concentrations in cellular compartments, monitoring ion fluxes, and visualizing protein trafficking processes [10-11]. Indeed, live cell imaging has now extensively been employed to study the biology and pathophysiology of *P. falciparum* in infected erythrocytes [12-13]. The unique favorable characteristics of erythrocytes allow attempts at the encapsulation of fluorescent probes with the capability of being entrapped within the cells [14].

Although prior studies had been performed on the encapsulation of a variety of biopharmaceuticals in erythrocytes as cellular carriers [14-15], the application of resealed erythrocytes containing high molecular weight dextran-linked fluorescent markers to track and dissect the parasite's metabolic processes has exposed new paradigms [16]. This has evoked plenty of research projects with the goal of using the potential capabilities of these resealed erythrocytes in different experimental situations such as in the study of pH regulation of the parasite's digestive vacuole [17-18], the mechanisms of action and resistance of antimalarial drugs [19], the cell attachment and invasion by the parasite [16] and the hemoglobin uptake and transport within parasite-infected erythrocytes [4, 20].

While observing these processes in parasite-infected erythrocytes by time-lapse based imaging is important, this technique was until recently not fully addressed for blood stages of *P. falciparum*, the causative agent of the most severe form of human malaria. Here, we provide a detailed description of the procedure for live cell imaging in blood stages of this important pathogen to study the uptake of host cell's cytoplasm and transport to the digestive vacuole, which is regarded as the terminal compartment of the endocytic process. The advent of live cell fluorescence imaging technology coupled with the ability to introduce fluorescent probes to erythrocytes provides new tools to study the dynamic endocytic process as it is thought to be essential for parasite survival and might represent a vulnerable target for chemotherapeutic efforts.

MATERIALS AND METHODS:

Materials: Tetramethylrhodamine-dextran (TMR-dextran, 25 mg, Life Technologies, No. D-1816) was purchased locally. Other chemicals and solvents were from chemical lab or HPLC purity grades, as needed, and purchased locally.

Blood samples: O^+ type peripheral blood samples (10 mL) were collected using 21-gauge 1.5" needles from informed consent healthy volunteers aged from 20 to 30 years that had no known diseases or blood-related diseases (i.e. HIV, hepatitis B or C, and malaria), took no medications, and were healthy by history and physical examination. Healthy volunteers were recruited at Blood Transfusion Unit, Hospital Universiti Sains Malaysia, Malaysia. Specimens were equally distributed to 3-mL purple-top tubes (K_2EDTA; Becton Dickinson), green-top tubes (heparin; Becton Dickinson) and blue-top tubes (3.2% sodium citrate; Becton Dickinson). Specimens were gently inverted eight times according to the manufacturer's instructions to ensure mixing of anticoagulant with blood, labeled and prepared for analysis.

Wet mounts and Giemsa-stained thin blood smears: The morphological changes of erythrocytes were observed before and after washing by centrifugation (121 × *g*, 5 minutes) with red cell wash (10 mM sodium phosphate, 160 mM NaCl, pH 7.4) as well as after 12-hour storage at 4°C in their initial vacutainer tubes. One volume (10 µL) of the samples was diluted into 50 volumes (500 µL) of red cell wash before preparing wet mounts or thin blood smears stained with Giemsa stain. Slides from EDTA, heparin and sodium citrate samples were observed using 100x magnification and oil immersion with constant light intensity. Areas that are well-populated with erythrocytes were selected (200-300 cells/field). A total of 1000 erythrocytes was evaluated and categorized according to the morphology of the cells as normal or altered. Normal erythrocytes are defined as having a round and biconcave disc shape, while altered erythrocytes (echinocytes) have regular broader-based short blunt projections of the cell membrane and lacking of central pallor.

Preparation of resealed erythrocytes: A modified hypotonic dilution method described by Dluzewski et al. [21] was used for loading human erythrocytes with TMR-dextran. In order to obtain resealed erythrocytes that retain minimal hemoglobin contents for parasite growth while allowing fluorescent probe entrapment into erythrocytes, the optimum ratio of packed erythrocytes to hemolysis buffer volume was determined.

For this purpose, 300 µL of washed packed erythrocytes were hemolyzed in ice-cold hemolysis buffer (5 mM sodium phosphate, pH 7.5, supplemented with 1 mM adenosine 5'-triphosphate magnesium salt, Mg-ATP) at different ratios (1:2, 1:3 and 1:4). Cell suspensions were mixed gently by 10 times inversion to ensure even hemolysis of the erythrocytes. After 10 minutes, cell suspensions were removed from the ice and cells were resealed by rapid addition of NaCl to a final concentration of 0.15 M (from a stock concentration of 5 M) followed by gentle mixing of the suspensions by several inversions. Resulting mixtures were incubated for 45 minutes at 37^{o}C on orbital shaker to ensure even resealing of the erythrocytes. At this point, the total amount of hemoglobin in the samples was measured by transferring a 40 µL aliquot of the cell suspensions into a respective well containing 110 µL hemolysis buffer and two-fold diluted across 96-well microtiter plates.

The total amount of hemoglobin was measured on a microplate reader (Bio-Rad, USA) by reading the absorbance at 415 nm and calculated using the ε of 125 $mM^{-1}cm^{-1}$. After 45 minutes, cell suspensions were spun down (3000 × *g*, 5 minutes) and supernatants were collected at this point and processed as mentioned above to measure the amount of hemoglobin loss after resealing. The cells were further washed three times until a clear supernatant was obtained. To measure hemoglobin retained in resealed erythrocytes, a 40 µL aliquot of the cell pellets was processed and analyzed as mentioned above. Pellets containing resealed erythrocytes were collected, resuspended in red cell wash or RPMI 1640 medium, wrapped in foil, and kept at 4^{o}C for later use.

Culturing of malaria parasites: Parasites (3D7 and D10 strains of *P. falciparum*) were cultured using O^{+} type human erythrocytes and pooled serum (Blood Transfusion Unit, Hospital Universiti Sains Malaysia, Malaysia) as described previously [22]. Parasite-infected erythrocytes were maintained in complete culture medium containing RPMI 1640 medium (GIBCO BRL, Invitrogen) supplemented with GlutaMAX I, 0.25% Albumax, hypoxanthine (0.21 mM, Sigma), 45% glucose (Sigma, G-8270), 50 mg/mL gentamicin (Duopharma, 42491-M) and 40 mL human serum. For synchronization, parasite pellets (~5% parasitemia, mainly ring stage) were suspended with 10 cell pellet volumes of 5% D-sorbital (Sigma) [23]. After incubation at room temperature for 10 minutes to lyse mature stage parasites, samples were centrifuged (270 × *g*, 5 minutes) and pellets were resuspended in complete culture medium and incubated at 37˚C under normal culture conditions (in a humidified atmosphere of 98% N_2, 1% CO_2 and 1% O_2). Parasites were allowed to mature for 24 hours.

Culturing of *P. falciparum* in resealed erythrocytes: To initiate infection of resealed erythrocytes, synchronized mature stage parasites were harvested on a Percoll gradient [24] at 24-28 hours post-synchronization. Harvested parasites were adjusted to 3% parasitemia (2% hematocrit) with complete culture medium and added to resealed erythrocytes with (T-RBC) or without TMR-dextran (R-RBC). Normal erythrocytes (N-RBC) were used as controls. Parasites were cultured in 6-well plates under normal culture conditions. Medium was changed twice each day. Susceptibility of resealed cells to invasion was determined and expressed as an invasion index. The invasion index is defined as the ratio of ring stage parasites present after incubation (24 hours after the addition of harvested parasites to resealed erythrocytes) to the original parasitemia (3%) (t=0). Giemsa-stained smears were examined by light microscopy and 500 cells were counted to determine the efficiency of invasion. The growth of the parasites was monitored throughout the 48-hour life cycle.

Imaging of parasite endocytic processes: Live cell imaging of parasite-infected erythrocytes containing TMR-dextran, a fluorescent marker of the endocytic process, was performed at 37°C using a 100x oil immersion objective (1.4 NA) on an inverted confocal microscope (Zeiss LSM 510). The confocal microscope was equipped with an incubation chamber and a heated stage maintained under an atmosphere of 5% CO_2 in air. Round glass coverslips (Fisherbrand) were cleaned before being mounted in culture chambers (Warner Instruments). The coverslips were then coated with concanavalin A (0.05 mg/mL, Sigma-Aldrich) for 5 minutes and rinsed twice with water and once with complete culture medium.

Cell suspensions (0.05% hematocrit) were placed onto the culture chambers and left for ~5 minutes to settle down. Parasite endocytic structures were visualized in 150 cells and analyzed using NIH Image J software. Similar analysis of parasite endocytic processes was also performed using an epifluorescence microscope (Olympus BX41) by using pre-coated slides with concanavalin a prior observation.

Statistical analysis: All experiments were carried out in triplicate (n=3) on three independent occasions and analyzed using SPSS version 20 (SPSS Inc., Chicago, Illinois, USA). All values used in analysis are presented as mean ± SD (standard deviation). Comparisons among different groups were performed by one-way analysis of variance (ANOVA) and differences were considered significant when $p<0.05$.

RESULTS:

Effects of different anticoagulants on the morphology of erythrocytes: Fig. 1 shows the mean percentage of normal (gray) and altered (black) erythrocytes measured before and after washing procedures as well as after cells were kept for 12 hours in their original tubes containing heparin, EDTA and sodium citrate, respectively. The mean percentages of all parameters determined from heparin samples compare well with those from EDTA and sodium citrate samples (marked with an asterisk).

Majority of the erythrocytes collected in heparin- and EDTA-containing tubes maintained their normal morphology and showed relatively low percentages of altered erythrocytes (echinocytes). Meanwhile, significant changes are evident on the morphology of erythrocytes collected in sodium citrate tubes ($p<0.05$). Effects were more noticeable for erythrocytes kept for 12 hours in the same sodium citrate tubes in comparison to those obtained from heparin and EDTA samples ($p<0.05$).

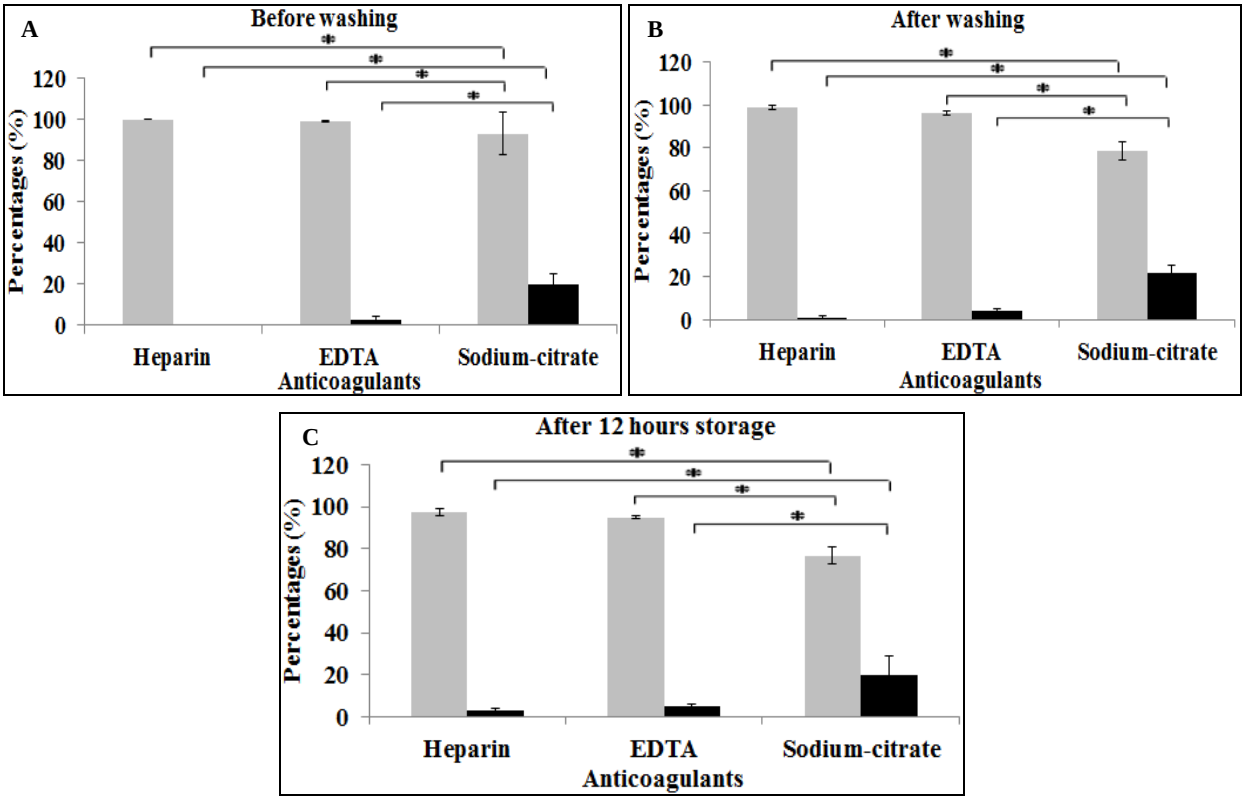


FIG. 1: PERIPHERAL ERYTHROCYTES WERE COLLECTED AND TRANSFERRED EQUALLY INTO HEPARIN-, EDTA- AND SODIUM CITRATE-CONTAINING TUBES. ALTERATIONS IN THE MORPHOLOGY OF ERYTHROCYTES WERE MONITORED (A) BEFORE AND (B) AFTER WASHING STEPS TO REMOVE THE BUFFY COAT AND (C) AFTER CELLS WERE KEPT FOR 12 HOURS IN THEIR INDIVIDUAL TUBES. THE MEAN PERCENTAGES OF NORMAL (GRAY) AND ALTERED (BLACK) ERYTHROCYTES WERE STATISTICALLY COMPARED BETWEEN SAMPLES AND PROCEDURES. VALUES REPRESENT MEAN ± S.D OF 300 CELLS IN EACH GROUP FROM THREE INDEPENDENT EXPERIMENTS PERFORMED IN TRIPLICATE. A SIGNIFICANT DIFFERENCE (P<0.05) BETWEEN SAMPLES IS INDICATED BY *

Effects of different ratios of packed erythrocytes to hemolysis buffer volume on loading parameters: To evaluate the effects of changes in hypotonic dilution method variables on the TMR-dextran loading efficiency, two indices were defined as loading parameters in this study: hemoglobin loss from and retained within resealed erythrocytes. The mean percentage of hemoglobin loss and hemoglobin retention within resealed erythrocytes is shown in **Fig. 2**. As the volume of hemolysis buffer used was increased, the loss of hemoglobin from erythrocytes increased significantly while the retention of hemoglobin decreased ($p<0.05$). These amounts of hemoglobin loss and retention, being practically feasible, permitted partial removal of hemoglobin while introducing the fluorescent probe, TMR-dextran into erythrocytes.

The morphology of resealed erythrocytes maintained a round biconcave shape as comparable to normal erythrocytes, however, a wider surface area of central pallor (double arrows) was observed as a result of hemoglobin release. At a 1:4 ratio, an even distribution of TMR-dextran labelling within the erythrocytes was observed as judged by examination with confocal microscopy, however a significant loss of hemoglobin (41.22 ± 7.74%) was measured rendering the erythrocytes unsuitable for parasite growth. At a ratio of 1:2, on the other hand, the amount of hemoglobin loss (26.29 ± 8.88%) during resealing was significantly less and the distribution of labelling within the erythrocyte population was acceptable. As an even distribution of the label and an optimum level of hemoglobin content were desired, a 1:3 ratio of packed erythrocytes to hemolysis buffer volume was used throughout all experiments.

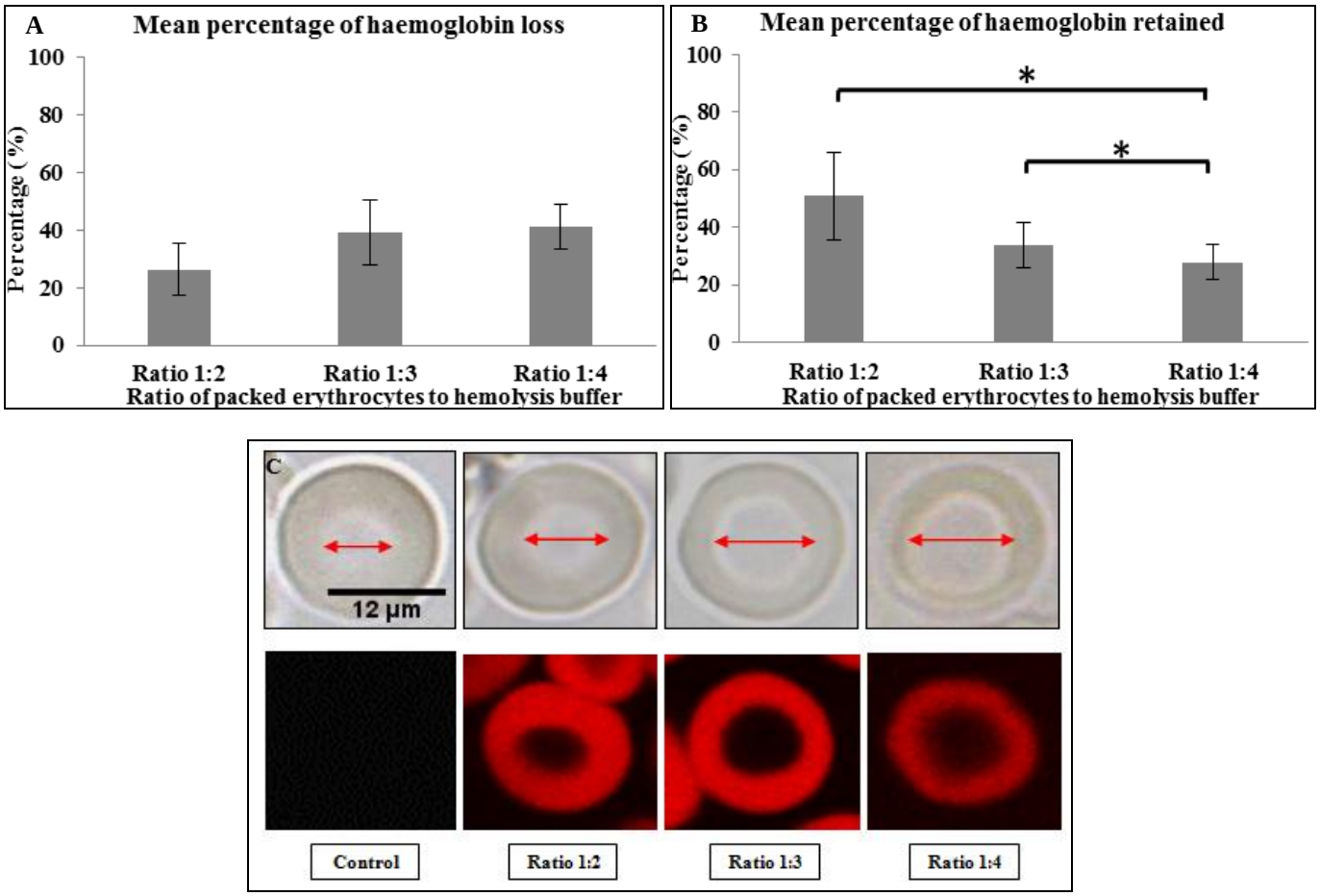


FIG. 2: (A) THE MEAN PERCENTAGE OF HEMOGLOBIN LOSS FROM AND (B) HEMOGLOBIN RETENTION WITHIN RESEALED ERYTHROCYTES WAS MEASURED FROM SAMPLES OF DIFFERENT RATIOS OF PACKED ERYTHROCYTES: HEMOLYSIS BUFFER VOLUME. A SIGNIFICANT DIFFERENCE ($P<0.05$) BETWEEN SAMPLES IS INDICATED BY *. (C) ALTHOUGH THE MORPHOLOGY OF RESEALED ERYTHROCYTES WAS MAINTAINED IN COMPARISON TO NORMAL ERYTHROCYTES, AN INCREASE IN THE CENTER PALLOR OF THE CELLS (DOUBLE ARROWS) WAS OBSERVED AS THE HEMOLYSIS BUFFER VOLUME USED WAS INCREASED. THE DATA REPRESENT THE MEAN ± S.D OF THREE INDEPENDENT EXPERIMENTS. SCALE BAR: 12 µM.

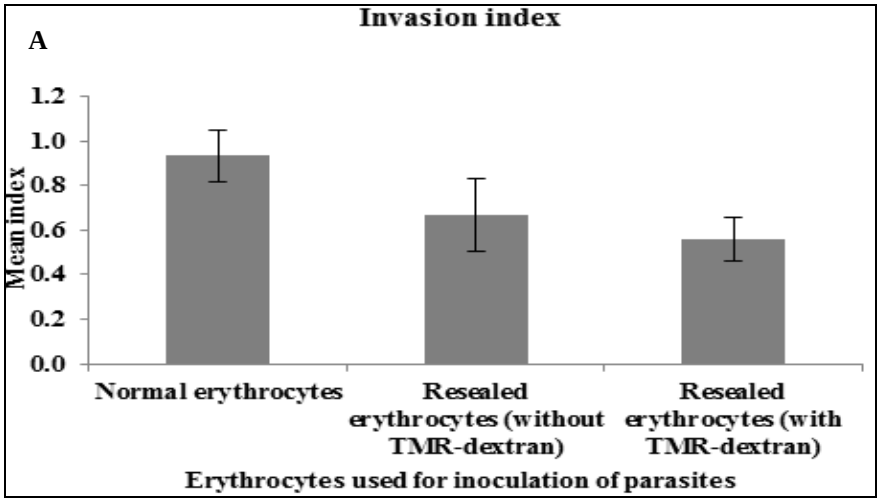
The growth and development of *P. falciparum* in resealed erythrocytes: The ability of the parasites to invade resealed erythrocytes with TMR-dextran (50 μM loading concentration) supplemented with 1 mM Mg-ATP was investigated. To initiate infection, purified mature parasites that were tightly synchronized were added into resealed erythrocytes with TMR-dextran using an optimized ratio of packed cells to hemolysis buffer volume (1:3). Normal and resealed erythrocytes without TMR-dextran were used as controls. Results were expressed as the invasion index, which is the ratio of the ring stage parasitemia (at 14 hours post-invasion) to the initial schizont stage parasitemia (3%, at t=0). As shown in **Fig. 3A**, released merozoites invaded normal and unlabeled resealed erythrocytes with invasion efficiencies of 0.93 ± 0.12 and 0.67 ± 0.17, respectively.

Although erythrocytes resealed in the presence of TMR-dextran were invaded with lesser efficiency (0.56 ± 0.10), the parasites were able to grow to maturity and completing the intraerythrocytic life cycle despite the reduced hemoglobin content of the resealed erythrocytes.

Live cell imaging using confocal microscopy (**Fig. 3B**) as well as epifluorescence microscopy assessment (**Fig. 3C**) also revealed the endocytic process of the parasites developing in TMR-dextran-labelled erythrocytes. Early endocytic structures were first observed at mid ring stage parasites aged 12 hours post-invasion based on the estimated time after invasion or inoculation, and had no visible hemozoin by examining the DIC image (**Fig. 3Biii** and **3Ciii,** first column). In some of these infected erythrocytes, the parasite was observed as a dark region in the fluorescence image with no evidence for endocytic structures (**Fig. 3Bii** and **3Cii,** second column). The fluorescent marker was concentrated in small peripheral compartments of the parasites (**Fig. 3Biii-iv,** white arrows), and the fluorescent signal in these endocytic vesicles was more intense than in the erythrocyte cytoplasm as the parasites grew to trophozoite stages (**Fig. 3Bv-vii,** second column**)**.

This suggests that the endocytosis of the host cell cytoplasm is followed by a process that concentrates the contents of the endocytic vesicles. Indeed, some hemoglobin-containing endocytic compartments in late ring stage parasites were observed to be undergoing the first step of hemozoin formation (**Fig. 3Biv,** blue arrows). As the parasite matures, these small endocytic structures were observed close to the digestive vacuole (**Fig. 3Bv-vii,** yellow arrows). Many of these structures were seen to be combined into a central digestive vacuole of the trophozoite stage parasites (marked by the presence of hemozoin in DIC images.

There was evidence of a large TMR-dextran-labelled feature within many ring and trophozoite stage parasites in the fluorescence images (**Fig. 3C,** green arrows). The fluorophore appeared to be at a similar concentration to the bulk of the erythrocyte cytoplasm. This structure might represent the invagination of the host cell cytoplasm into the region of the parasite and could correspond to a proposed macropinocytic structure described by Elliott et al. [5]



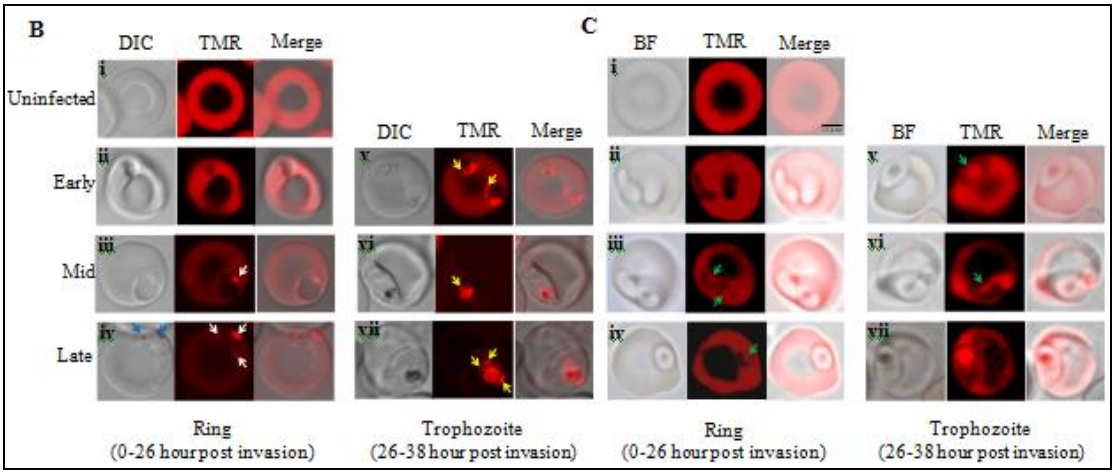


FIG. 3: (A) EFFICIENCY OF INVASION OF RESEALED ERYTHROCYTES BY *P. FALCIPARUM*. THE ENDOCYTIC PROCESS OF THE MALARIA PARASITES IN RESEALED ERYTHROCYTES INCORPORATED WITH TMR-DEXTRAN AS AN ENDOCYTIC MARKER AS OBSERVED BY (B) LIVE CELL IMAGING USING CONFOCAL MICROSCOPY AND (C) EPIFLUORESCENCE MICROSCOPY. SCALE BAR: 13 µM.

DISCUSSION: Encapsulation of biochemical substances into erythrocytes leads to the production of resealed erythrocytes that are widely used in biopharmaceutical studies as carriers [14-15]. In malaria research, resealed erythrocytes have been used to study the physiological functions of the digestive vacuole pH [17-18], the mechanisms of antimalarial drug action and resistance [19], the parasite attachment to and invasion of host cells [16, 25-26], and the formation of the digestive vacuole [4]. Several research groups have developed methods for trapping high molecular weight dextran-linked fluorescent markers in resealed erythrocytes such as hypotonic hemolysis (hypotonic dilution, hypotonic pre-swelling, hypotonic dialysis and isotonic osmotic lysis techniques), chemical perturbation of the membrane, electro insertion and entrapment by endocytosis [18-19, 27-28].

In this present study, the entrapment of TMR-dextran into erythrocytes was done by using hypotonic dilution technique, which is based on reversible swelling of erythrocytes in hemolysis buffer solution. This technique is the fastest and simplest method for loading low-to-high molecular weight compounds [27]. An optimum ratio of packed erythrocytes to hemolysis buffer volume (1:3) resulted in nearly 40% of hemoglobin loss. The release of hemoglobin content gives sufficient space for the encapsulation of TMR-dextran [29]. Despite lacking of hemoglobin, the parasites were able to grow and develop within the resealed erythrocytes, which were still retained more than 30% of the original hemoglobin.

Studies have suggested that resealed erythrocytes must retain more than 20% of the original hemoglobin content for efficient growth and invasion of the malaria parasite [21, 30-31].

Minor alterations of the method allowed the preparation of resealed erythrocytes, the majority of which showed morphology closely similar to that of normal erythrocytes, although an increase in the surface area of central pallor of the resealed erythrocytes was observed. This is expected since the loss of hemoglobin during the method of TMR-dextran loading in the erythrocytes resulted in changes in the cell morphology. Sprandel et al. have suggested that mechanical force during sample processing such as vigorous mixing, excessive centrifugal force, prolonged fixed angle centrifugation or re-centrifugation of sample tubes also need to be considered [32].

Furthermore, previous study reported that the type of storage containers can significantly affect cell morphology as well as cause hemolysis during storage [33], therefore, the present study compared different anticoagulant collection tubes in regard to the morphological parameters and to evaluate the latter reliability and effectiveness for use in the resealing technique.

Our findings showed that blood samples collected in EDTA tubes maintained normal cell morphology before and after washing procedures as well as after 12 hours storage in the same tubes at 4°C.

This observation was in agreement with Antwi-Baffour et al., which reported a normocytic and normochromic profile of erythrocytes collected in EDTA [33]. Considering heparin is expensive and can interfere with the staining properties of cells, EDTA tubes were become the anticoagulant of choice.

Tiffert et al. have shown that the efficiency of invasion of resealed erythrocytes by the parasites was slightly reduced as the cell morphology had changed [34]. The present study revealed that, with the ATP supplement (1 mM), the resealed erythrocytes were able to support the merozoites entry despite the lower invasion index compared to the normal erythrocytes, and also capable in supporting of the parasite growth [4, 16, 21]. The addition of ATP into a hemolysis buffer has been shown to be critical for parasite invasion [16, 26]. No invasion occurred when an analogue of ATP, adenylyl-imidodiphosphate (AMP-PNP), was substituted for ATP [21]. Other evidence suggests that the ATP supplement is needed to maintain the cell's normal shape and morphology [31, 35].

Previous ultrastructural studies of the feeding mechanism of *P. falciparum* employed thin-section electron micrographs [36-37]. There is a need for alternative methods to examine the endocytic process of live and intact cells under less-disruptive conditions [38-39]. In the present study, the uptake and transfer of hemoglobin by the parasites was monitored by using live cell confocal and epifluorescence microscopies. Both techniques were able to visualize the parasite endocytic process although the confocal microscopy provided much higher contrast and excluded out-of-focus lights in the images [40]. The findings revealed that the fluorescent marker, TMR-dextran was observed in small peripheral structures at the mid ring stage. Similar results were obtained by Abu Bakar et al. [4], which very early stage parasites developing in SNARF-1-dextran labelled erythrocytes showed no endocytic compartments; however, at the mid ring stage, the fluorescent marker was observed in small peripheral structures.

They reported the presence of several small vesicles at the parasite periphery that contained hemozoin microcrystals, which we observed in late ring stage parasites that likely to represent the first compartments formed during the formation of the digestive vacuole. As the parasite matures, many of these vesicles were seen adjacent to the digestive vacuole. A larger structure within ring and trophozoite stage parasites was often observed and could represent the recently described "big gulp" by Elliot et al. [5].

However, selective photobleaching assays performed by Abu Bakar et al. [4] showed that this large structure remains connected to the erythrocyte cytoplasm and temporally co-exists with the peripheral vesicles in which hemoglobin digestion is occurring. This suggests the formation of several small peripheral compartments as the earliest event in the digestive vacuole formation.

CONCLUSION: In conclusion, the erythrocytes collected using EDTA tubes were loaded successfully with TMR-dextran with the practically acceptable loading parameters. The resealed erythrocytes incorporated with TMR-dextran were evaluated with respect to their potential as a model for the endocytic process of blood stage parasites. The data from this study provide evidence for the endocytic structures and pathway in both ring and trophozoite stage parasites. TMR-dextran proved the ability as an indicator of the endocytic process. Given its general availability, widespread use and relatively low cost, a salt of EDTA was become the anticoagulant of choice, which maintained the normal morphology of collected erythrocytes for use in the endocytic study of the parasite.

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CONFLICT OF INTEREST: The authors report no conflict of interest.

REFERENCES:

1. de Koning-Ward TF, Dixon MW, Tilley L and Gilson PR. *Plasmodium* species: master renovators of their host cells. Nature Review Microbiology 2016; 14(8): 494-507.

2. Tilley L, Dixon MW and Kirk K. The *Plasmodium falciparum*-infected red blood cell. International Journal of Biochemistry and Cell Biology 2011; 43(6): 839-42.
3. World Health Organisation (2015). World Malaria Report 2015. Retrieved July, 18, 2016, from http://www.who.int/ malaria/ publications/world-malaria-report-2015/ report/ en/
4. Abu Bakar N, Klonis N, Hanssen E, Chan C and Tilley L. Digestive-vacuole genesis and endocytic processes in the early intraerythrocytic stages of *Plasmodium falciparum*. Journal of Cell Science 2010; 123(3): 441-50.
5. Elliott DA, McIntosh MT, Hosgood HD, Chen S, Zhang G, Baevova P and Joiner KA. Four distinct pathways of hemoglobin uptake in the malaria parasite *Plasmodium falciparum*. Proceedings of National Academic of Science U.S.A 2008; 105(7): 2463-2468.
6. Lazarus MD, Schneider TG and Taraschi TF. A new model for hemoglobin ingestion and transport by the human malaria parasite *Plasmodium falciparum*. Journal of Cell Science 2008; 121(11): 1937-1949.
7. Gulati M, Narula A, Vishnu R, Katyal G, Negi A, Ajaz I and Lumb V. Plasmepsin II as a potential drug target for resistant malaria. The Delhi University Journal of Undergraduate Research and Innovation 2015; 1(3): 85-95.
8. Myers KA and Janetopoulosa C. Recent advances in imaging subcellular processes. F1000Research 2016; 5: 1553.
9. Terai T and Nagano T. Fluorescent probes for bioimaging applications. Current Opinion in Chemical Biology 2008; 12(5): 515–521.
10. Malucelli E, Fratini M, Notargiacomo A, Gianoncelli A, Merolle L, Sargenti A, Cappadone C, Farruggia G, Lagomarsino S and Iotti S. Where is it and how much? Mapping and quantifying elements in single cells. Analyst 2016; 141(18): 5221-5235.
11. Pak YL, Swamy KM and Yoon J. Recent progress in fluorescent imaging probes. Sensors 2015; 15(9): 24374-24396.
12. Weiss GE, Gilson PR, Taechalertpaisarn T, Tham WH, de Jong NW, Harvey KL, Fowkes FJ, Barlow PN, Rayner JC, Wright GJ and Cowman AF. Revealing the sequence and resulting cellular morphology of receptor-ligand interactions during Plasmodium falciparum invasion of erythrocytes. PLOS Pathogens 2015; 11(2) e1004670.
13. Rohrbach P. Imaging ion flux and ion homeostasis in blood stage malaria parasites. Biotechnology Journal 2009; 4(6): 812-825.
14. Chen JL, Dhanaliwala AH, Dixon AJ, Farry JM, Hossack JA and Klibanov AL. Acoustically active red blood cell carriers for ultrasound-triggered drug delivery with photoacoustic tracking. In Ultrasonics Symposium (IUS), 2015; IEEE International 2015 (pp. 1-4). IEEE
15. Villa CH, Cines DB, Siegel DL, and Muzykantov V. Erythrocytes as Carriers for Drug Delivery in Blood Transfusion and Beyond. Transfusion Medicine Reviews 2016.
16. Zuccala ES, Satchwell TJ, Angrisano F, Tan YH, Wilson MC, Heesom KJ and Baum J. Quantitative phospho-proteomics reveals the *Plasmodium* merozoite triggers pre-invasion host kinase modification of the red cell cytoskeleton. Scientific Report 2016; 6: 19766.
17. Abu Bakar N. Measuring pH of the *Plasmodium falciparum* digestive vacuole by flow cytometry. Tropical Biomedicine 2015; 32(3): 485-493.
18. Saliba KJ, Allen RJ, Zissis S, Bray PG, Ward SA and Kirk K. Acidification of the malaria parasite's digestive vacuole by a H+-ATPase and a H+-pyrophosphatase. Journal of Biological Chemistry 2003; 278(8); 5605-5612.
19. Hayward R, Saliba KJ and Kirk K. The pH of the digestive vacuole of *Plasmodium falciparum* is not associated with chloroquine resistance. Journal of Cell Science 2006; 119(Pt 6): 1016-1025.
20. Klonis N, Crespo-Ortiz MP, Bottova I, Abu-Bakar N, Kenny S, Rosenthal PJ and Tilley L. Artemisinin against *Plasmodium falciparum* requires hemoglobin uptake and digestion. Proceedings of the National Academy of Sciences of the United States of America 2011; 108(28): 11405-11410.
21. Dluzewski AR, Rangachari K, Wilson RJM and Gratzer WB. A cytoplasmic requirement of red cells for invasion by malaria parasites. Molecular and Biochemical Parasitology 1983; 9(2): 145-160.
22. Trager W and Jensen JB. Human malaria parasite in continuous culture. Science 1976; 193(4254): 673-675.
23. Lambros C and Venderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. Journal of Parasitology 1979; 65: 418-420.
24. Miao J and Cui L Rapid isolation of single malaria parasite–infected red blood cells by cell sorting. Nature Protocols 2011; 6(2): 140-146.
25. Dluzewski A, Wilson R and Gratzer W. Relation of red cell membrane properties to invasion by *Plasmodium falciparum*. Parasitology 1985; 91(02): 273-280.
26. Rangachari K, Dluzewski A, Wilson R and Gratzer W. Cytoplasmic factor required for entry of malaria parasites into red blood cells. Blood 1987; 70 (1): 77-82.
27. Soundararajan M, Thandamaraikannan S, Palaniswamy S and Subbiah L. A review on resealed erythrocytes as a novel drug delivery system. Asian Journal of Pharmaceutical and Clinical Research 2015; 8(4): 101-107.
28. Ventakesh E, Aparna C, Umasankar K, Reddy PJ and Prabhakaran V. Resealed erythrocytes: a novel approach to treat chronic diseases. International Journal of Pharmaceutical Sciences Review and Research 2013; 23(2): 298-306.
29. Zade-Oppen A, Ostling S and Marsden N. On how macromolecules reduce hemoglobin loss in hypotonic hemolysis. Upsala Journal of Medical Sciences 1976; 84(2): 155-161.
30. Goldberg DE, Slater AFG, Beavis R, Chait B, Cerami A and Henderson GB. Hemoglobin degradation in the human malaria pathogen *Plasmodium falciparum*: a catabolic pathway initiated by a specific aspartic protease. The Journal of Experimental Medicine 1991; 173 (4): 961-969.
31. Olsen JA. and Kilejian A. Involvement of spectrin and ATP in infection of resealed erythrocyte ghosts by the human malaria parasite *Plasmodium falciparum*. The Journal of Cell Biology. 1982; 95(3): 757-762.
32. Sprandel U, Clark JB, Hubbard AR and Chalmers RA. Morphology of haemoglobin-containing human erythrocyte 'ghosts'. Micron. 1981; 12(1): 29-36.
33. Antwi-Baffour S, Quao E, Kyeremeh R and Mahmood SA. Prolong storage of blood in EDTA has an effect on the morphology and osmotic fragility of erythrocytes. International Journal of Biomedicine Science and Engineering 2013; 1(2): 20-23.
34. Tiffert T, Lew VL, Ginsburg H, Krugliak M, Croisille L and Mohandas N. The hydration state of human red blood cells and their susceptibility to invasion by *Plasmodium falciparum.* Blood 2005; 105(12): 4853-4860.

35. Aryee NA, and Takakuwa Y. Modification of the mechanical properties of red blood cell membrane by spent Plasmodium falciparum culture supernatant. Jordan Journal of Biological Sciences 2013; 6(1): 25-30.
36. Bannister LH, Hopkins JM, Fowler RE, Krishna S and Mitchell GH. A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages. Parasitology Today 2000; 16(10): 427-433.
37. Milani KJ, Schneider TG and Taraschi TF. Defining the morphology and mechanism of the hemoglobin transport pathway in *Plasmodium falciparum*-infected erythrocytes. Eukaryotic cell 2015; 14(4): 415-426.
38. Lanzer M and Rohrbach P. Subcellular pH and Ca^{2+} in *Plasmodium falciparum*: Implications for understanding drug resistance mechanisms. Current Science 2007; 92(11): 1561-1570.
39. Wissing F, Sanchez CP, Rohrbach P, Ricken S and Lanzer M. Illumination of the malaria parasite *Plasmodium falciparum* alters intracellular pH. Implications for live cell imaging. Journal of Biological Chemistry 2002; 277(40): 37747-37755.
40. Sanderson MJ, Smith I, Parker I, and Bootman MD. Fluorescence microscopy. Cold Spring Harbor Protocols 2014; 10: pdb-top071795.

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