In-Depth Biological Alteration Analysis of *Plasmodium Knowlesi*-Infected Red Blood Cells Using A Non-Invasive Optical Imaging Technique

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Research

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Abstract

Background

Imaging techniques are commonly used for understanding disease mechanisms and their biological features in such a microenvironment of the cell. In recent work, the understanding of parasite biology of malaria *Plasmodium knowlesi* was compiled from functional in vitro and imaging analysis using serial block-face scanning electron microscopy (SEM). Sample fixation during SEM analysis possibly makes the parasite membrane destruction.

Methods

In this study, we used a non-invasive optical technique, tomography, to explore the biological changes during the development of infected red blood cells (RBCs) by *P. knowlesi*. The commercial common-path diffraction optical tomography was used to measure individual infected RBCs' three-dimensional refractive index tomogram without an additional dyeing agent.

Results

We simultaneously examined structural, chemical, and physical modification by a parasite inside of host RBCs. The results had similar patterns with previous studies using SEM and other parasites such as *P. falciparum* and *Babesia*. Interestingly we found that *P. knowlesi* did not use hemoglobin as much as *P. falciparum* as a source of nutrition.

Conclusions

This tomography technique opens new ways to study the pathophysiology of malaria parasites more efficiently and is considered a promising tool for studying each *Plasmodium* species more in-depth.

Background

Malaria pathophysiology is still an interesting topic to be explored. A variety of *Plasmodium* species with an extensive variant of each feature makes malaria still hard to be eradicated. In addition, immune evasion and drug resistance to malaria have been touched by multidisciplinary expertise [1–3]. Imaging techniques have been used for a long time in malaria pathophysiology studies. It has been continuously developed since the discovery of classical light microscope to exceptionally sophisticated technology such as Förster resonance energy transfer (FRET), fluorescence lifetime imaging microscopy (FLIM), third-harmonic generation (THG), two-photon absorption fluorescence (2PAF) imaging, and common-path diffraction optical tomography (cDOT) [4].

Tomography, one of the non-invasive optical techniques, has been used to explore the biological changes during the development of *P. falciparum* and *Babesia* parasites-infected red blood cells (RBCs) [5, 6]. The tomography principle uses optical interferometry or digital holography, which is non-invasive, label-free, and quantitative. In addition, cDOT is a recent technique that can measure both the three-dimensional structure and dynamics of live cells simultaneously [6, 7].

Out of 6 *Plasmodium* species that infect humans, *P. knowlesi* is considered to be a threat pathogen. It is recognized as zoonotic malaria and has started to infect humans, particularly in Malaysia, and spread out to other areas in Southeast Asia. Besides, *P. knowlesi* also showed a symptom like cerebral malaria as found in *P. falciparum* [8, 9]. During the host cell invasion, the parasite modifies the host cell environment to support their life, leads to morphological, biochemical, and mechanical modification [10, 11].

Recently *P. knowlesi* topography study applied for structural observation using serial block-face scanning electron microscopy [12]. However, that technique required a particular treatment like fixation before analysis. In addition, it has a limitation in determining live dynamics of parasite and host cells because of the fixation and metal coating steps [6, 13, 14]. Instead of using an additional treatment, our study focuses on live cells for biological analysis. Thus, the cells are kept in the natural form without losing a parasite membrane during observation. Tomography is considered an easy and fast technique for individual cell qualitative and quantitative analyses.

This investigation used cDOT of individual *P. knowlesi*-infected RBCs to investigate the alteration of morphological, biochemical, and mechanical parameters in every asexual developmental stage parasites. This research has shown that all parameters could be quantified as described in our data, including volume, surface area, sphericity, hemoglobin content, and membrane fluctuation. We also compared the data to *P. falciparum* as the most malignant human malaria parasites. From all data, we found that the hemoglobin consumption of *P. knowlesi* was less than *P. falciparum* as a source of nutrition. In the end, this method provides an extremely simple and accessible for studying malaria pathophysiology.

Methods

Parasite culture

P. knowlesi A1.H1 adapted human RBCs was cultured in RPMI 1640 media (Invitrogen Life Technologies, Grand Island, NY) supplemented by horse serum (Gibco, Life Technologies), L-glutamine, 25 mM HEPES (Invitrogen Life Technologies), and 0.5% Albumax II (Invitrogen Life Technologies). The culture was maintained at 2% hematocrit with mixed gas (90% N₂, 5% CO₂, and 5% O₂) at 37°C while *P. falciparum* was cultured with slightly different supplementation as described elsewhere [15]. Both parasites were treated with gentamicin to prevent contamination.

Schizont synchronization

The synchronization of parasite stages was conducted by setting a schizont as an initial stage. *P. knowlesi* schizonts were isolated using 50% nycodenz, while *P. falciparum* used magnetic-activated cell sorting (MACS) system [16]. Purified schizont was re-culture at 2% parasitemia, the ring stage of *P. knowlesi* was observed 10 hrs after the invasion, while *P. falciparum* needed 18 hrs [17, 18].

Analysis of parasite-infected human RBCs alteration

Three to five percent parasitemia of each parasite stage was set for further analysis using a commercial 3D cDOT system (HT-1H, Tomocube Inc., Republic Korea). The parasite was diluted with Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium, then apply to 25 ´ 50 mm coverslip (Matsunami Glass Ind., Osaka, Japan). The data was visualized using Tomo Studio software (Tomocube Inc., Daejeon, Republic Korea) and analyzed quantitatively by RBCs characterization software using a specific algorithm [19]. Fifteen samples of each stage were used for further analysis.

Statistical analysis

GraphPad Prism (GraphPad Software, San Diego, CA) was used to analyze data statistically. The student's *t*-test was applied to determine differences between the two group data. The data with $p < 0.05$ were considered to have significant differences.

Result

Three-dimensional refractive index maps of malaria-infected RBCs

Data of refractive index (RI) generated a 3-dimensional optical tomogram that was utilized for analyzing various inside and outside features of cells [20]. The specific developmental blood stage of the malaria parasite was observed after synchronization (Fig. 1). The RI of the healthy RBC following maturation of *P. knowlesi* parasite stage was gradually decreased with their mean values for healthy RBCs (1.375 ± 0.005), ring (1.370 ± 0.004), trophozoite (1.369 ± 0.004), and schizont (1.367 ± 0.004) stages. The value could be more understandable by comparing RI and phase map images of each developmental stage (Fig. 2). Healthy RBCs are described as donuts shapes due to their biconcave shape. The dark red color showed the highest value, meanwhile, the yellow color showed the lowest RI. The uniformity of healthy RBCs content was described as uniform color, most of the RBCs containing 95% spherical protein like hemoglobin [21]. Whereas the parasitized RBCs seen have a gradient color affected by parasite-filled RBCs space. The yellow color exhibited parasite cytoplasm, while the dark red indicated hemozoin. The pattern also was observed in *P. falciparum* RI with their values for ring (1.373 ± 0.004), trophozoite (1.371 ± 0.003), and schizont (1.367 ± 0.004) stages.

Morphological alteration of *P. knowlesi*-RBCs in different stages

During infection, the parasite growth induced host cell constituents deficiency [22]. It is reflected by morphological changes of infected RBCs, including the RBCs shape that was becoming more spherical from the initial biconcave or donuts shape [23]. Decreasing cellular volume and surface area of the RBCs also were observed in the infected RBCs. The shape alteration of infected-RBCs showed in the mean value of sphericity which gradually increased from healthy (0.616 ± 0.103), ring (0.723 ± 0.090), trophozoite (0.721 ± 0.099), and schizont (0.770 ± 0.041) (Fig. 3A) stages. In terms of mean values of cytosol volume for healthy RBCs (86.686 ± 7.619 fL), ring (80.413 ± 9.331 fL), trophozoite (76.084 ± 8.282 fL), and schizont (71.177 ± 11.490 fL) stages (Fig. 3C). Decreasing that value was caused by the parasite occupied and digesting the nutrition provided in the RBCs cytosol. With the increasing of RBCs sphericity during parasite maturation, the surface area of the RBCs continuously decreased for healthy RBCs (154.863 ± 16.924 μm^2), ring (150.802 ± 12.270 μm^2), trophozoite (150.009 ± 16.916 μm^2), and schizont (142.176 ± 23.840 μm^2) stages (Fig. 3D). A similar pattern of cellular volume and surface area of *P. falciparum* also could observe as following data 76.632 ± 18.553 , 71.075 ± 17.240 , 63.395 ± 15.388 fL (cellular volume), and 76.632 ± 18.553 , 71.075 ± 17.240 , 63.395 ± 15.388 μm^2 (surface area) for healthy RBCs, ring, trophozoite, and schizont stages, respectively.

Alteration of biological dynamics in asexual stages of *P. knowlesi*-infected RBCs

Parasite actively proliferates, and growth utilizes an environment inside RBCs. It affects an alteration of the structural and live dynamics of host RBCs [24]. RBC membrane fluctuation was used to identify live dynamics alteration during infection. The data showed that increasing the mean value of fluctuation due to maturation of parasite for healthy RBCs (78.330 ± 23.051 nm), ring (114.634 ± 31.060 nm), and schizont (237.643 ± 266.212 nm), however, the trophozoite (107.395 ± 35.443 nm) slightly decreases (Fig. 4A).

Hemoglobin Content In Each Stage Of Parasite-infected Rbcs

The implication of a significant decrease of cytosol volume in the *P. knowlesi*-infected RBCs was obviously shown in decreasing hemoglobin content following the maturation of the parasite. Hemoglobin is a major content of cytoplasm, and it was used by the parasite as the nutrient for their growth and proliferation [25, 26]. The more merozoite exists inside RBCs, and the more hemoglobin will decrease. The mean value of hemoglobin content was observed from the healthy (29.143 ± 4.103 pg), ring (24.623 ± 3.154 pg), trophozoite (23.446 ± 2.913 pg), and schizont (22.003 ± 3.913 pg) stages (Fig. 4B). However, *P. falciparum*-infected RBCs were calculated in the ring (19.203 ± 4.666 pg), trophozoite (16.941 ± 4.137 pg), and schizont (14.597 ± 3.541 pg) stages, which reflects *P. falciparum* more actively consumed hemoglobin than *P. knowlesi*.

Discussion

Once malaria parasites entered and started to take over the RBCs, their morphology is actively changed due to their growth and development. Malaria parasites transform during the maturation from immature ring and trophozoite to mature schizont stages. The environment within the host RBCs that majorly consist of protein cytoplasm or hemoglobin will decrease as well as parasites use it for their metabolism [27]. In addition, the loss of the donuts shape causes a decrease in the surface area of red blood cells and makes their shape more spherical, which affects the loss of deformability. [23, 28].

The parasites use hemoglobin as a precursor of amino acid synthesis [29]. Hemoglobin is degraded to the toxic product called hemozoin then converts to a structural compound called hemozoin to avoid the reactive oxygen species [30, 31]. Hemozoin produces high contrast imaging; some microscopy was observed, such as polarization microscopy, dark-field microscopy, and resonance Raman microscopy [4, 32, 33]. The schizont refractive index also exhibited it was significantly increased because of hemozoin production. It is also affected by many proteins released out during merozoite egress [34].

A major protein released during the schizont stage has a photolytic function such as cytoskeleton-degrading malarial proteases, falcipain-2 and plasmepsin II, plus a family of putative papain-like proteases called serine repeat antigen [34, 35]. It gave a consequence to have a high fluctuation in the RBCs membrane. Besides, several excess nutrients are transferred by several pathways like new permeability pathways studied by several groups, especially in *P. falciparum* [36]. Meanwhile, the ring stage has a more considerable fluctuation value, probably because it still requires an arrangement of a new host RBC environment.

Our study focused on the RBCs as an object, while previous research focused on the parasite alteration itself [12]. This study also comparable with previous research that used a similar technique in *P. falciparum* and *Babesia* parasites. Our study found that the uniqueness of *P. knowlesi* tended not to make the significant alteration within-host RBCs compare to *P. falciparum* in hemoglobin content. The fact that hemoglobin content of *P. knowlesi*-infected RBCs was higher than *P. falciparum*, might occur with the following assumptions: (1) *P. knowlesi* life cycle shorter than *P. falciparum*; thus, *P. knowlesi* did not have enough time to consume the hemoglobin. (2) Host preference differences. *P. knowlesi* is used to stay in the monkey RBCs but should use human RBCs may affect parasite metabolism. (3) The parasite might use another source more than hemoglobin. However, those hypotheses needed further study.

The level of cytosol volume of *P. falciparum*-infected RBCs was more drastically decreased than *P. knowlesi*. The clinical data has strengthened this assumption where *P. knowlesi* patients hemoglobin amount was higher than *P. falciparum* with the following described for *P. knowlesi* (9.7 g/dL) and *P. falciparum* (7.15 g/dL) [37]. This study could be used in the understanding mechanism of parasites utilize the host environment. In addition, our finding leads to a possibility that *P. knowlesi* treatment against hemoglobin metabolism may not affect much more than *P. falciparum*.

Conclusion

In conclusion, the application of cODT opens a new way that much easier to study the pathophysiology of malaria by exploiting the live sample using the refractive index. This study supports that each *Plasmodium* species has a particular profile in utilizing host resources that correlate to different pathways of those species carry on their lives.

Abbreviations

SEM

Scanning electron microscopy; RBCs:Red blood cells, FRET:Förster resonance energy transfer, FLIM:Fluorescence lifetime imaging microscopy, THG:Third-harmonic generation (THG); 2PAF:Two-photon absorption fluorescence; cDOT:Common-path diffraction optical tomography; *P. falciparum*:*Plasmodium falciparum*; *P. knowlesi*:*Plasmodium knowlesi*; MACS:Magnetic-activated cell sorting; DPBS:Dulbecco's phosphate-buffered saline; RI:refractive index.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee, Kangwon National University Hospital, ROK (IRB No. 2014-08-008-002). Informed consent was obtained from all of the participants

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Author contributions

E.R.F and E.-T. H. designed the study. E.R.F, F.M, J.-H P., and S.-K. L. conducted the laboratory work and data analysis. S.-H. N., W.-S. P., J.-H. H., and K.-W. H. supported materials and data analysis. All authors contributed to writing, reading, and approving the manuscript.

Availability of data and material

The datasets supporting the conclusions are included in the article.

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Figures

Fig. 1

Figure 1

Morphology of malaria parasite during synchronization. (A-C) *Plasmodium knowlesi* culture, (D-F) *P. falciparum* culture. (A and D) mixed stages of parasites, (B and E) synchronized schizont stage, and (C and F) newly invaded ring stage.

Fig. 2

Figure 2

Three-dimensional refractive index and phase map of *P. knowlesi*-infected RBCs. (A-B) 3D RI maps of *P. knowlesi*-infected RBCs and (C-D) phase map image. The black arrow indicates hemozoin.

Fig. 3

Figure 3

Reflective index and morphological profile of *P. knowlesi*-infected RBCs. (A) refractive index, (B) sphericity, (C) cellular volume, and (D) surface area of *P. knowlesi*-infected RBCs.

Fig. 4

Figure 4

Membrane dynamic and biochemical alteration of *P. knowlesi* and *P. falciparum*-infected RBCs. (A) Fluctuation of each stage in *P. knowlesi*-infected RBCs and (B) hemoglobin content both *P. knowlesi*- and *P. falciparum*-infected RBCs.

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