รายงานการวิจัยฉบับสมบูรณ์

โครงการ การแสดงออกของ P-glycoprotein และความสัมพันธ์กับปริมาณของ cytokines ในผู้ป่วยมาลาเรียขึ้นสมอง

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โครงการ

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Expression of P-glycoprotein and correlations with cytokines in cerebral malaria patients

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Abstract

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P-glycoprotein is an efflux protein, expressed on several cell types, including vascular endothelial cells at the blood-brain barrier (BBB). Function of P-gp at BBB is to prevent entry of potentially toxic molecules into brain parenchyma. Cerebral malaria is a complication in central-nervous system (CNS), found in patients with severe malaria. This is caused by infection of *Plasmodium falciparum*. In cerebral malaria (CM), several changes in CNS are developed, resulting in neurological symptoms, death and, possibly, neurological sequelae. In the present study, we assessed expression of P-gp at protein levels in postmortem brain samples. Five parts of brain, cerebellum, cerebral cortex, brain stem, hippocampus and striatum, were collected from seven subjects, which were divided into three groups; 1) CM (n=3), 2) encephalopathy with no infection of *P. falciparum* (n=1) and 3) other causes with no brain involvement (control, n=3). Brain-capillary endothelial cells were isolated and analyzed for P-gp expression by using SDS-PAGE and western blotting techniques. We observed that P-gp expression in brain-capillary endothelial cells decreased in striatum collected from all three CM subjects, compared to control samples. However, the expression in cerebellum was reduced in two of three CM subjects and only one CM subject showed the reduction in brain stem, hippocampus and cerebral cortex. In the encephalopathy subject, P-gp expression was reduced in cerebellum, cerebral cortex, brain stem and striatum, but not in hippocampus. These results demonstrated that expression of P-gp can be modulated in CM and encephalopathy, but the modulation is dissimilar in different parts of brain.

Keywords: cerebral malaria, P-glycoprotein, encephalopathy, brain capillary, endothelial cell

บทคัดย่อ

P-glycoprotein (P-gp) เป็นโปรตีนชนิด efflux protein ซึ่งพบอยู่บนเซลล์หลายชนิด รวมถึงเซลล์บุผนังหลอดเลือดที่ blood-brain barrier (BBB) หน้าที่ของ P-gp ที่ BBB ได้แก่การป้องกันไม่ให้โมเลกุลที่อาจทำให้เกิดพิษเข้าสู่เนื้อสมอง มาลาเรียขึ้นสมองเป็นภาวะแทรกซ้อนในสมองส่วนกลางที่พบในมาลาเรียขั้นรุนแรง ภาวะนี้เกิดจากการติดเชื้อมาลาเรียชนิด *Plasmodium falciparum* ในภาวะมาลาเรียขึ้นสมอง พบว่ามีการเปลี่ยนแปลงเกิดขึ้นหลายอย่างในสมองส่วนกลางทำให้เกิดอาการทางประสาท การเสียชีวิตและอาจรวมถึงผลกระทบต่อระบบประสาทที่อาจพบหลังจากการหายของโรค ในศึกษานี้ มีการประเมินการแสดงออกของ P-gp ระดับโปรตีนในตัวอย่างเนื้อเยื่อสมองที่เก็บหลังจากการเสียชีวิต โดยมีการเก็บสมองห้าส่วน (cerebellum, cerebral cortex, brain stem, hippocampus และ striatum) จากผู้เข้าร่วมการวิจัย จำนวน 7 ราย ซึ่งแบ่งเป็น 3 กลุ่ม คือ 1) ผู้ที่เป็นมาลาเรียขึ้นสมอง (n=3), 2) ผู้ที่เป็น encephalopathy โดยไม่มีการติดเชื้อ *P. falciparum* (n=1) และ 3) ผู้ที่ไม่มีการแทรกซ้อนในสมอง (กลุ่มควบคุม, n=3) เซลล์บุผนังหลอดเลือดฝอยในสมองถูกแยกออกมาเพื่อการวิเคราะห์ระดับของการแสดงออกของ P-gp โดยใช้วิธี SDS-PAGE และเทคนิค western blotting เราพบว่าการแสดงออกของ P-gp expression ในเซลล์บุผนังหลอดเลือดฝอยในสมองลดลงในสมองส่วน striatum ที่แยกมาจากผู้ที่เป็นมาลาเรียขึ้นสมองทั้ง 3 ราย โดยเทียบกับกลุ่มควบคุม ในขณะที่เซลล์บุผนังหลอดเลือดฝอยในสมองมีการแสดงออกของ P-gp ลดลงใน cerebellum เพียง 2 รายจาก 3 ราย และใน brain stem hippocampus และ cerebral cortex พบการลดลงของ P-gp เพียง 1 ราย สำหรับกลุ่มที่เป็น encephalopathy โดยไม่มีการติดเชื้อ *P. falciparum* พบว่าการแสดงออกของ P-gp ลดลงในเซลล์บุผนังหลอดเลือดฝอยในสมองที่แยกจาก cerebellum, cerebral cortex, brain stem และ striatum แต่ไม่ลดลงใน hippocampus ผลการศึกษานี้แสดงให้เห็นถึงการแสดงออกของ P-gp ที่เปลี่ยนไปในมาลาเรียขึ้นสมอง ซึ่งมีรูปแบบที่แตกต่างกันในแต่ละส่วนของสมอง

Executive Summary

Background: P-glycoprotein is an efflux protein, expressed on several cell types, including vascular endothelial cells at the blood-brain barrier (BBB). Function of P-gp at BBB is to prevent entry of potentially toxic molecules into brain parenchyma. Cerebral malaria is a complication in central-nervous system (CNS), found in patients with severe malaria. This is caused by infection of *Plasmodium falciparum*. In cerebral malaria (CM), several changes in CNS are developed, resulting in neurological symptoms, death and, possibly, neurological sequelae. The aim of this study is to assess expression of P-gp in CM.

Methods: Brain samples (cerebellum, cerebral cortex, brain stem, hippocampus and striatum) were collected from subjects which were divided into three groups; 1) CM (n=3), 2) encephalopathy with no infection of *P. falciparum* (n=1) and 3) other causes with no brain involvement (control, n=3). Brain-capillary endothelial cells were isolated from each samples and analyzed for expression of P-gp by using SDS-PAGE and western blotting techniques

Results: We observed that P-gp expression in brain-capillarry endothelial cells decreased in striatum collected from all three CM subjects, compared to control samples. However, the expression in cerebellum was reduced in two of three CM subjects and only one CM subject showed the reduction in brain stem, hippocampus and cerebral cortex. In the encephalopathy subject, P-gp expression was reduced in cerebellum, cerebral cortex, brain stem and striatum, but not in hippocampus.

Summary and discussion: Expression levels of P-gp were changed in CM and encephalopathy subjects. Even though the patterns were not identical among samples from different subjects, these changes suggests importance of P-gp in pathoohysiology of CM and encephalopathy.

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Chapter 1: Introduction

I. Malaria and Cerebral malaria

1. Introduction

Malaria is a parasitic disease caused by infection of protozoa in the genus Plasmodium. There are four species of Plasmodium that infect human, *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovalae*. The first two species are more common than the other two. Malaria causes important public-health problems, including mortality rate and expenses used for transmission control and disease treatment.

Endemic areas of malaria are in tropical and subtropical regions where the climate enables the parasite and its vector to transmit the disease. Endemic areas cover about 107 countries and territories (1), including those in Africa, Asia, the Middle East, Central and South America. Approximately 41% of world's populations live in endemic areas, and there are about 700,000 to 2.7 million people died of malaria each year and most of them were children (2). Even though several countries could reduce malaria transmission, malaria cases and death were still reported. Moreover, endemic areas are likely to expand because of the change in the World's climate, so called the global-warming phenomenon (3).

Among those countries in endemic areas, Thailand is also in tropical area with reports of malaria cases. Malaria is usually found along the borders between Thailand and our neighbor, especially on the Thai-Myanmar border. While malaria in the central part of the country can be eliminated, that on the border is severe. This is because of highly efficient vectors, enhanced vector life span and extensive migration into and out of these areas. Main difficulties impeding the disease control include transmission at the border among foreign workers, drug resistance of the parasite, high fatality rates among non-immune people such as tourists and migrants, education challenge and extensive migration into and out of these areas, resulting in a new epidemic area (4).

Generally, when patients are infected with malaria parasite, they develop fever as the parasites are in the blood circulation and the fever drops when the parasites are out of the blood circulation, but in erythrocytes. In addition to fever, malaria patients develop symptoms such as headache, malaise, nausea, muscular pain, and mild diarrhea.

Malaria is transmitted by mosquitoes in the genus Anopheles. In addition, blood transfusion and transmission via placenta were also reported. Malaria's life cycle is found in two hosts, mosquitoes in genus *Anopheles* and human (Figure 1.1). The parasite can replicate by both asexual and sexual reproduction. First, the parasites in sporozoite stage enter human body by injection from mosquitoes and infect hepatocytes. Then, they replicate asexually in hepatocytes, developing into schizonts in which contain several merozoites. After that there are ruptures of infected hepatocytes, and merozoites are released and enter blood circulation. Then, merozoites infect erythrocytes and develop into trophozoites and schizonts in which contain several merozoits. The parasite stages in erythrocytes are called erythocytic schizogony. After the rupture of erythrocytes, merozoites are released and infect new erythrocytes. Some of the merozoites commit to sexual development in erythrocytes, leading to male (microgametocytes) and female (macrogametocytes) gametocytes. When a mosquito bites a malaria-infected patient, gametocytes are taken with blood meal. There are then gametogenesis and fertilization, causing ookinete. After that, ookinete penetrates mosquito's midgut wall and develop into oocyst. Mature oocysts contain sporozoites, and when oocyst ruptures, sporozoites are released and penetrate salivary gland. When the infected mosquito takes another blood meal, sporozoites are injected with saliva and infect another human host.

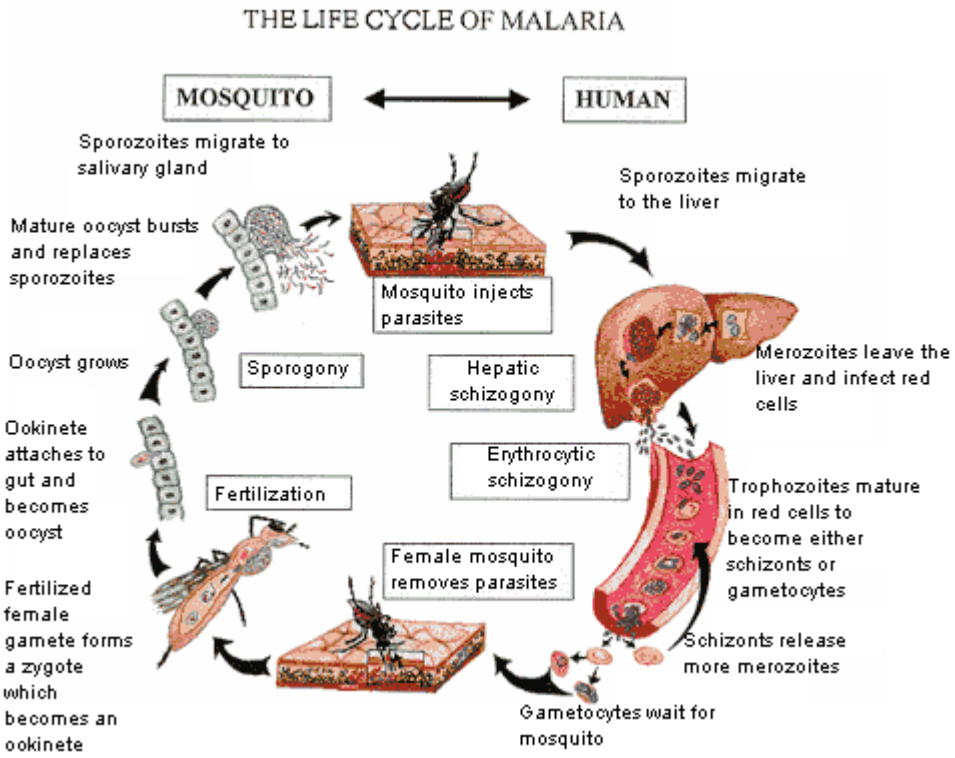


Figure 1.1. Drawing showing life cycle of malaria in human and anopheles mosquito hosts

Source: http://myweb.stedwards.edu/jdoming/malaria.htm

2. Infection of *Plasmodium falciparum*

P. falciparum is the protozoa parasite, whose infection causes malaria and can lead to more severe condition called severe malaria. This condition is defined by the presence of asexual stage of *P. falciparum* in blood circulation with a possible fatal manifestation or complication. The fatality rate of *P. falciparum* is about 1%, accounting for about 1 – 3 million death around the world, and 80% of the death are caused by cerebral malaria. There are reports of *P. falciparum* infection in 300 to 500 million people and the death of over a million people (5).

There are three predisposing factors of severe malaria; 1) parasite, 2) host and 3) geographic and social factors. First, parasite factors are drug resistance, multiplication rate, invasion pathway, cytoadherence, rosetting, antigenic polymorphism, antigenic variation and malaria toxin. Next, host factors include immunity (non-immune, immunosuppressed and immunocompromised patients), splenectomy, pre-existing organ failure, proinflammatory cytokines, genetics, age and pregnancy. Finally, geographic and social factors consist of access to treatment, cultural and economic factors, political stability and transmission intensity (6, 7). According to WHO, symptoms and complications observed in severe malaria include impaired consciousness, jaundice, extreme weakness, hyperpyrexia, hyperparasitemia, coma, severe anemia, metabolic acidosis, renal failure, pulmonary edema (ARDS) respiratory distress, hypoglycemia, circulatory collapse, spontaneous bleeding, hemoglobinemia, acidosis, repeated convulsion, hypotension and shock, fluid, electrolyte or acid-base disturbance, hemoglobinuria, vomiting of oral drugs and other indicators of poor diagnosis (8).

2.1. Cerebral malaria

Cerebral malaria (CM) is a complicated condition found in patients with severe malaria, which is caused by *Plasmodium falciparum*. This is a specific condition because it has an involvement of central-nervous system (CNS), characterized by impaired consciousness. Diagnostic criteria of CM, defined by WHO, include parasitemia with *P. falciparum,* coma, exclusion of other causes of coma and favorable response to antimalaria therapy (9). Clinical manifestations usually start with fever and headache, followed by drowsiness, confusion, impaired consciousness, seizure and unarousable coma (Glasgow coma scale < 7/15, Table 1.1), anemia, bleeding, renal failure, pulmonary edema, metabolic acidosis, hypoglycemia, hypovolemia, hyperpyrexia, brain stem signs, and subsequent death (10, 11). In addition, involvements of other organs are present, that are an enlargement of liver and spleen

(hepatosplenomegaly). Moreover, some patients who are recovered from CM, especially children, may suffer from neurological sequelae, such as speech and behavioral difficulties.

2.2. Neurological histopathology studies of CM

Autopsy studies of CM show several macroscopic abnormalities, including dusky discoloration of the grey matter, opacity of the leptomeninges, brain edema, congestion and petechial hemorrhage. Histopathology examinations of CM demonstrated obstruction of brain capillaries and venules with parasited and non-paresited red blood cells. Postmortem studies report swollen brain, petechia, ring and spotty hemorrhage and granulomatous nodule (Dürck's granuloma). Even though no damage of the endothelial cells is seen at the microscopic level, immunohistochemistry studies showed activation of endothelial cells (12) and disruption of blood-brain barrier (13).

Table 1.1. Glasgow Coma Scale (GCS)

Eyes -opening	Example	Score
Spontaneous	Opens eyes on own	4
To Voice	Opens eyes when asked to in a loud voice	3
To Pain	Opens eyes when pinched	2
No Response	Does not open eyes	1

Verbal Response (Talking)	Example	Score
Orientated	Carries on a conversation correctly and tells examiner where he is, who he is, and the month and year	5
Confused Conversation	Seems confused or disoriented	4
Inappropriate Words	Talks so examiner can understand him but makes no sense	3
Sounds	Makes sounds that examiner cannot understand	2
No Response	Makes no noise	1

Motor Response	Example	Score
Commands	Follows simple commands	6
Localizes Pain	Pulls examiner's hand away when pinched	5
Withdraws from Pain	Pulls a part of body away when pinched	4
Abnormal Flexion	Flexes body inappropriately to pain	3
Abnormal Extension	Body becomes rigid in an extended position when examiner pinches him	2
No Response	Has no motor response to pinch	1

Note: Total score = E + V + M. For example, GCS 9 = E2 V4 M3 and GCS $\leq$ 8, GCS 9 - 12 and GCS $\geq$ 13 is considered as severe, moderate and minor condition, respectively.

2.2. Pathophysiology of cerebral malaria

Several hypotheses are generated based on clinical and laboratory observations to explain pathophysiology of CM, such as mechanical, cytokine (humoral), permeability, intravascular coagulation, metabolic-effect hypotheses. However, no single hypothesis can clearly explain clinical symptoms and consequences of CM. Even though several studies have been shown evidences supporting these hypotheses in both human and animal models, actual causes of death in CM patients are not yet clearly explained. It is believed that there are several involved factors. Interestingly, while other pathogens (bacteria and viruses), that infect central nervous system and cause neurological dysfunction, can penetrate into brain parenchyma by increasing the barrier's permeability and by direct invasion (14), the *P. falciparum* malaria stays in the vascular space without entering the brain parenchyma.

There are three important hypotheses have been used to explain pathophysiology of CM, as following

1) Mechanical hypothesis

The mechanical hypothesis states that pathophysiology of CM involves cytoadherence (sequestration) of malaria-infected erythrocytes on vascular endothelial cells in the brain microvasculature. Malaria-infected erythrocytes can either bind to each other, forming agglutation, or bind to non-infected erythrocytes, forming rosetting, and adhere to vascular endothelial cells. The sequestration is known as a cause of neurological complications in *P. falciparum* infection. This event leads to reduction of microvascular blood flow, which helps promote malaria growth and prevent malaria from destruction by the reticuloendothelial system. As blood flow is reduced, resulting in vascular occlusion; therefore, tissues and organs with reduced blood flow lack of nutrients, especially glucose, and oxygen to maintain their energy levels (metabolism) and, finally, loss their function. Moreover, the malaria parasites produce toxin interfering host metabolism.

Evidences show that *P. falciparum*-infected erythrocytes adhere on vascular endothelial cells by using *P. falciparum* erythrocyte membrane protein (PfEMP-1) and receptors on microvascular endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1), E-selectin and CD36. In addition, adhesion of the malaria-infected red blood cells on vascular endothelial cell can also induce expression of TNF-α and apoptosis of the endothelial cells by

mechanisms involving oxidative stress (15). These findings appear to represent linkage between mechanical and cytokine hypotheses (see below).

There is also a release of lactate in to cerebrospinal fluid (CSF) because of production of cerebral lactate. This represents anaerobic glycolysis which occurs when oxygen supply to CNS is limited, which may occur because sequestration and vascular obstruction or direct effect of the infection on metabolism. Therefore, it remains unclear whether this event occurs from mechanical or metabolic effect.

2) Cytotoxic (humoral) hypothesis

Cytokines are a group of small proteins, produced by immune cells when bodies encounter foreign bodies. These proteins have stimulating activities on several target cells, including immune cells as well as other cells such as vascular endothelial cells.

The cytokine hypothesis for CM pathophysiology was first initialized by studies by, among the others, Clark, et al. (16) and Brown et al. (17). Several cytokines have been reported to be released by induction of inflammatory responses to malaria-parasite antigen and its toxin. These include tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ), IL-1β, GMCSF, IL-2 and IL-10. Several researchers showed that the cytokines have roles on pathogenesis of CM in both human and animal models. In rhesus monkey model, infection with *Plasmodium coatneyi* induces expression of TNF-α, IFN-γ and IL-1β in cortex and cerebellum (18). In mouse model, infection of *Plasmodium berghei* ANKA (PbA) and *P. yoelii* 17 XL causes fatal cerebral malaria. Studies reported that cytokines including TNF-α and IFN-γ increase in murine CM (19). In TNF-α-deficient mouse, PbA cannot induce cerebral malaria. TNF-α is required for up-regulation of ICAM-1, NO, recruitment of mononuclear cells and platelets as well as damage of cerebral microvasculature (20, 21). In addition to TNF-α, IFN-γ is also important in development of CM (21). When IFN-γ-deficient mice is infected with PbA, upregulation of TNF-α and ICAM-1 is reduced. It has been shown that expression of TNF-α and IFN-γ increases in brain of PbA-infected mice and that there is infiltration of inflammatory cells as well as damage of microvasculature in murine brain with CM (19). TNF-α can also cause disruptions of synaptic transmission via production of nitric oxide (NO), leading to coma,

which is one of the characteristic of CM. In human studies, correlations between cytokines and CM were also reported. It was also reported that plasma levels of the cytokine have correlations with disease severity (22). This is also supported by evidences demonstrating that increases in cytokines in brain are related to CNS complications, CM (17, 23). From autopsy studies, TNF-α and ICAM-1 expressions were detected in brain CM (24). Study by Armah et al. also showed that cytokines TNF-α and IL-1β are well correlated with expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin (25). Moreover, cytokines can also induce changes in protein at blood-brain barrier (BBB) (26), representing to disruption of BBB and increases in its permeability. This study represents linkage between cytokine and permeability hypotheses.

ICAM-1 is an intercellular adhesion molecule which can be upregulated by TNF-α. One of TNF-α-target cells is endothelial cells, lining blood vessels and capillaries. Upregulation of adhesion molecules by cytokines helps facilitate the cytoadhesion of parasite-infected erythrocytes, monocytes (macrophages) and platelets on vascular endothelial cells of cerebral microvasculature (13), resulting in vascular occlusion as explained in the mechanical hypothesis. *In vitro* study showed that activation of human umbilical vein endothelial cells (HUVEC) by TNF-α helps increase cytoadhesion of malaria-infected red blood cells isolated from patients with *P. falciparum* infection (severe and cerebral malaria) (27). In mouse model, ICAM-1 is up-regulated when mice are infected with PbA (28). In addition, mice with an expression of ICAM-1 died more rapidly than those without ICAM-1 expression (29). ICAM-1-deficient mice also respond to PbA infection in lower extents than wild-type mice. Those responses include breakdown of blood-brain barrier, sequestration of malaria-infected red blood cells and macrophage and thrombocytopenia. (29). This supports ICAM-1's role in CM pathophysiology.

In human studies, increases in adhesion molecules (ICAM-1 and E-selectin) are observed in brain of patients who died of CM (12). Visualized by immunohistochemistry, ICAM-1 expression and parasite sequestration are co-localized. These represent roles of ICAM-1 as a mediator of cytoadhesion of malaria-infected red blood cells, which may auto-agglutinated or forming rosetting with uninfected red blood cells, leukocytes and platelets.

3) Permeability hypothesis: breakdown of blood-brain barrier (BBB)

Permeability hypothesis was originated from the finding that radioactive-labeled albumin can move across blood-brain barrier (BBB) in rhesus monkey infected with *P. knowlesi* (30). This movement causes by an increase in BBB's permeability. Subsequently, there are leakages of plasma and consequential brain edema. These events can be prevented by corticosteroids.

BBB consists of endothelial cells which are tightly bound together by protein interaction called tight junction. Function of BBB is to control transport of molecules and ions into and out of brain parenchyma. One important purpose is to prevent transport of toxic agents into brain and resulting neurotoxicity. Therefore, agents or drugs moving across BBB are limited. Any substances may move across BBB, depending on their sizes and charges by two major pathways: passive diffusion and ATP-driven, active transports. In addition, to limit passage of toxic agents into brain parenchyma, endothelial cells at BBB express efflux proteins which help remove substances out of brain parenchyma and prevent their possible neurotoxicity. The efflux proteins expressed at BBB include P-glycoprotein (P-gp, ABCB1) and MRP1 (ABCC1).

Pathological studies of human or animal models with CM showed breakdown of BBB and functional abnormalities in filtration property (31). Combes et al. (32) have shown that microparticles and platelets also induce changes in BBB. Consequently, plasma proteins, which normally are not able to move across BBB, can pass BBB and activate macrophages at the perivascular space. Activated macrophages then are activated, producing and secreting various substances, including cytokines such as TNF-α and IFN-γ. Subsequently, there is a breakdown of BBB, leakage of fluids in to brain and brain edema.

It has been suggested that breakdown of BBB in CM is caused by histamine secreted from immune cells, or production of reactive oxygen species (ROS) from activated neutrophils and hemoglobin digestion in malaria parasites (10, 33). Moreover, antimalarial agents can also induce oxidative stress. Medana et al. (34) have shown that there is oxidative stress at blood vessels and hemorrhage area in cerebral malaria patients.

In addition, adhesion molecule ICAM-1 is involved in disruption of tight junction of BBB in patients with cerebral malaria (13). The disruption involves changes in protein components at tight junctions which are ZO-1, occludin and vinculin (13). This study represents

relationships between cerebral malaria, expression of adhesion molecules and disruption of tight junction of BBB (31).

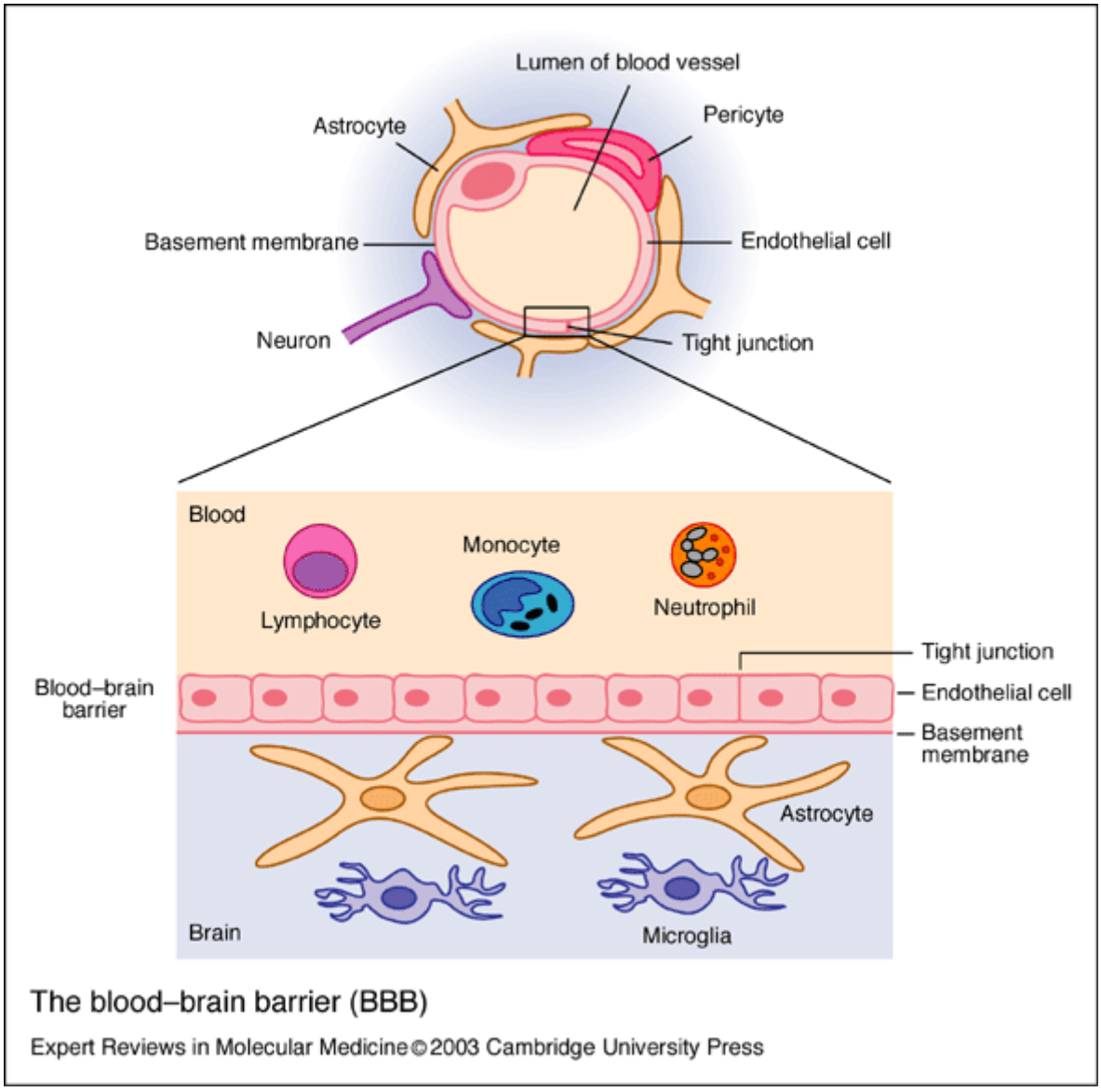


Figure 1.2. Schematic drawing represents components and structure of blood-brain barrier.

Source:

http://www.stanford.edu/class/humbio103/ParaSites2005/Ivermectin/images/blood%20brain%20barrier.gif

II. P-Glycoprotein

1. Introduction

P-glycoprotein (P-gp) is a glycoprotein, encoded from gene mdr1 and mdr3 in human and mdr1a, mdr1b and mdr2 in mice (35). However, only human mdr1 and mouse mdr1a encode proteins that function as efflux proteins. P-gp is a member of ATP-binding cassette (ABC) transporter superfamily, namely ABCB1. Proteins in this superfamily are transmembrane protein with ATP-binding domains. They also share a common function in transporting compounds across biological membrane by using energy from ATP hydrolysis.

P-gp is a glycoprotein with 1,280 amino acids and molecular weight of 170 kD (Figure 1.3). N-terminal and C-terminal are located at the intracellular side and there are two homologous halves with six hydrophobic transmembrane domains in each half, creating barrel-like structure conformation (36). There are two ATP-binding domains; one between 6^{th} and 7^{th} transmembrane domain and the other after 12^{th} transmembrane domain. P-gp has three glycosylation sites at the region between the 1^{st} and 2^{nd} transmembrane domain. In addition, there are several phosphorylation sites at the region between the 6^{th} and 7^{th} transmembrane domains. These sites are phosphorylated by cAMP-dependent protein kinase A and by protein kinase C. It is proposed that phosphorylation is a mechanism that P-gp activity is modulated (37). This is supported by a study showing induction of P-gp expression and activity by stimulation of protein kinase C (38).

2. Function of P-glycoprotein

Physiological function of P-gp is not yet clear, but it is accepted that P-gp has roles on responsiveness to drug treatment and prevention of tissue from potentially toxic agents. P-gp was initially recognized in tumor or cancer cells that do not respond to chemotherapy (drug resistance). Overexpression of P-gp in cancer cells have been shown to be associated with multidrug resistance. Those cancer cells include ovarian, breast, testis, and colon cancer as well as myeloma, lymphoma (39-42).

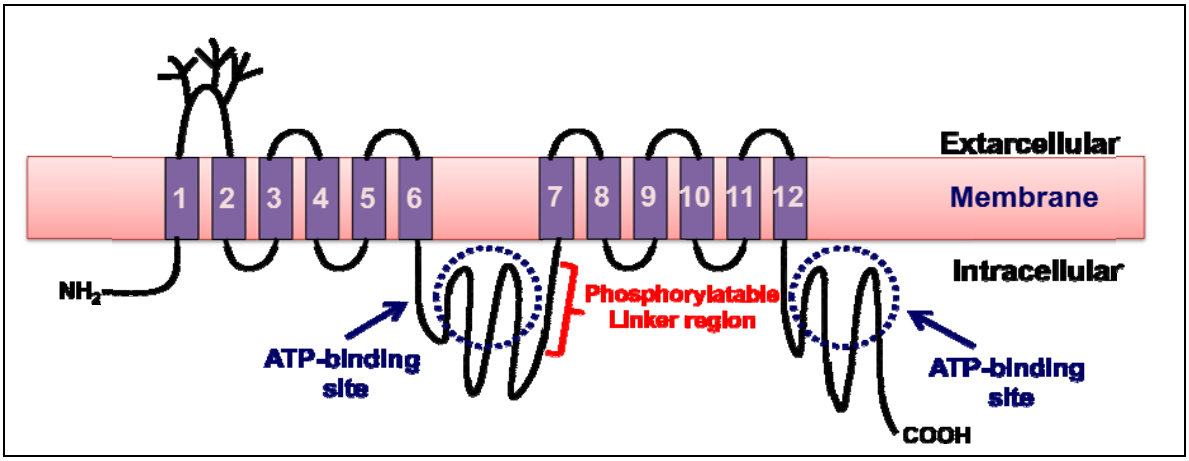


Figure 1.3. Schematic drawing of human P-glycoprotein transporter, showing two homolg halves, consisting of six transmembrane region in each half, two ATP-binding sites, three glycosylation sites and phosphorylatable linker region.

At the brain capillaries (BBB), P-gp function is to transport several lipophilic molecules entering endothelial cells back to the blood in order to prevent intracellular accumulation of harmful agents. Therefore, P-gp helps prevent brain from exposure to several lipophilic agents. These agents include drugs that are used to treat many central-nervous system diseases such as cerebral HIV infection and epilepsy. Understanding expression and mechanism of its function can improve efficiency of drug treatment for central-nervous system diseases.

3. P-gp substrates and reversal agents

Substrates of P-gp, agents being transported by P-gp, are a wide range of compounds with little structural relationships. They can be hydrophobic, amphipathic and natural compounds. As shown in table 1.2 (43-47), they include biological compounds, antitumor agents, cytotoxic agents, HIV protease inhibitors, antibiotics, immunosuppressive drugs, pesticides, calcium-channel blockers, anti-malarial drugs, opioids, calmodulin antagonists and other agents. It is interesting that some P-gp substrates are also P-gp reversal agents.

The P-gp reversal agents such as verapamil have been shown to help improve respond to chemotherapy, supporting roles of P-gp on drug efflux and resistance of tumor cells (48). Therefore, in order to improve responsiveness of cancer to anticancer therapy, several studies reported P-gp inhibitors (P-gp reversal agents). However, their toxicity is a major challenging. P-gp reversal agents include calcium-channel blockers, antihypertensive, antibiotics, immunosuppressants, steroid hormones (progesterone, corticosteroid), HIV protease inhibitors, alcoholism treatment drug and other agents (Table 1.2)

As mentioned earlier, antimalarial drugs are also P-gp substrates. Pham et al. (49) reported that racemic mefloquine and its enantiomers can be exported by P-gp in cultured cells. They demonstrated that inhibition of P-gp by P-gp reversal agents, verapamil, cyclosporin A or chlorpromazine, induces decreases in efflux of [^{14}C]-mefloquine. In addition, Barraud de Lagerie et al. (50) also reported brain uptake of mefloquine in human and rat is modulated by P-gp inhibitor. Interestingly, mefloquine inhibits activity of P-gp, which may be associated with neurological effect caused by mefloquine (51).

Table 1.2 Substrates and inhibitors of P-gp

Classification	**Substrates**	**Inhibitors (Reversal agents)**
Biological compounds	Bilirubin, steroid hormones	steroid hormones
Antitumor agents	Vinca alkaloids (vincristine, vinblastine), Anthracyclines (doxorubicin, daunorubicin, epirubicin), Epipodophyllotoxins (Etoposide, Teniposide), Paclitaxel (taxol), Actinomycin D, Topotecan, Mithramycin, Mitomycin C	Vinca alkaloids (vinblastine), Anthracyclines (doxorubicin, daunorubicin),
Cytotoxic agents	Colchicine, emetine, ethidium bromide, puromycin, cyclic and linear peptides, gramicidin D, valinomycin, N-acetyl-leucyl-leucyl-norleucine	-
HIV protease inhibitors	Ritonavir, indinavir, saquinavir, nelfinavir	Ritonavir, indinavir, saquinavir, nelfinavir
Antibiotics	Tetracyclin, erythromycin, rifampicin	Rifampicin, cefoperazone, ketoconazole, cephalosporins
Immunosuppressive drugs	Cyclosporine A, PSC 833 (valspodar)	Cyclosporine A, PSC 833 (valspodar)
Pesticides	Ivermectin	-
Calcium-channel blockers	Verapamil	Verapamil, dihydropyridines
Antihypertensives	-	Reserpine
Opioids	Morphine, β-endorphin	-
Antimalaria drugs	Quinolines (quinine, quinidine)	Quinolines (quinine, quinidine)
Calmodulin antagonists	Trifluoperazine	Trifluoperazine
Alcoholism treatment drug	-	Disulfiram
Other agents	Hoechst 33342, Rhodamine 123, Calcein-AM	Curcumin

4. P-gp: Mechanism of action

Several models have been proposed for mechanism of P-gp action; however it is not yet conclusive. It is clear that ATP binding and hydrolysis are required for P-gp to move its substrates from cytosolic compartment to extracellular space. The most favored model is the "flippase" model (52, 53). In this model, P-gp moves its substrates against concentration gradients by bind to its substrates at the inner leaflet of the lipid bilayer and flip them to the outer leaflet.

5. Expression of P-glycoprotein

P-gp is expressed on many cell types, including epithelia of kidney, liver, lung, and intestine as well as vascular endothelial cells of testis, ovary and blood-brain barrier (BBB) (54-57). In addition, P-gp is also expressed in human hematopoietic stem cells, cytotoxic T cells and natural killer cells (36).

At the BBB, P-gp, as an efflux protein, is expressed at the apical side of the endothelial cells. It has a role in controlling transport across BBB to prevent neurotoxicity (43, 58). Studies in mice lacking mdr1a (mice P-gp) have shown that these mice are more sensitive to neurotoxicity than normal mice, supporting roles of P-gp on neurotoxicity.

6. P-gp polymorphism

Studies have identified single nucleotide polymorphisms (SNPs) of P-gp. SNPs can alter P-gp expression and activity (59-61). In addition, SNPs are potentially important with influence on responsiveness to drugs in individuals (62). Neurological adverse effects of the anti-malarial drug mefloquine is also depend on P-gp polymorphism (63).

7. Modulation of P-gp expression and activity

Reports have shown that P-gp can be altered in various circumstances. Its expression is rapidly upregulated by extracellular and intracellular stress, including cytotoxic agents, heat shock, irradiation, genotoxic stress, inflammation, inflammatory mediators, cytokines, growth factors as well as conditions causing oxidative stress (64, 65). Reactive-oxygen species (ROS) are produced under oxidative-stress conditions, including hypoxia, inflammation, glucose deprivation, and metabolic disturbance (65). Felix et al. have shown that culturing rat brain endothelial cells under oxidative-stress condition (using with H_2O_2) results in induction of expression of P-gp. Hypoxia also induces increases in P-gp expression (66). This induction is mediated by which mechanisms involve activation of NFκB (67). In addition, Ledoux et al. (68)

have shown that hepatoma cells (Fao) being cultured in glucose-depleted media have increases in P-gp expression at mRNA and protein levels.

How ROS induce P-gp expression is complex. Nwazuzu et al. have shown that, in primary cultured rat brain endothelial cells, activation of extracellular-signal regulated kinases (ERK1/2), protein kinase C (PKC), the p46 isoform of stress-activated protein kinase (SAPK), c-Jun and Ark is involved in induction of P-gp expression by ROS (H_2O_2), while inhibition of NFκB augments increases in P-gp expression (69). Activation of protein kinase C can both increase P-gp expression and enhance P-gp activity (38).

Additionally, P-gp expression can be induced by xenobiotics and hormones (70). Several drugs can modulate P-gp function, as following;

1) Calcium channel blockers: Verapamil, Nifedipine, Bepridil, Nicardipine
2) Calmodulin antagonists Antibiotics: Trifluoperazine, Chlorpromazine, trans-Flupenthixol
3) Immunosuppressive drugs: Cyclosporine, Tacrolimus (FK506), Rapamycin, PSC-833
4) Antibiotics: Rifampicin, Tetracycline, Cefoperazone,
5) Steroidal agents: Progesterone, Tamoxifen, RU-486
6) Surfactant: Tween-80, Cremophor-EL, Solutol HS 15
7) Quinolines: Chloroquine, Quinidine, Quinine
8) Other Drugs: Reserpine, Yohimbine, Aminodarone, Dipyridamole, Terfenadine

Many cytokines can modulate either expression or activity of P-gp. It has been shown that cytokines, interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), TNF-α, TGF-β, IFN-γ and leukoregulin suppress expression of P-gp (71, 72). In contrast, other studies show that TNF-α can induce P-gp expression (73, 74).

In addition to changes in levels of P-gp expression, P-gp can be also functionally modulated. It has been shown that TNF-α can decrease function of P-gp in caco-2 cells, primary rat hepatocytes and BB19 brain capillary endothelial cells without effects on P-gp expression (75). In addition, study using rat brain capillaries have shown decreases in P-gp transport by treatment of TNF-α and lipopolysaccharide (LPS) (76). Interestingly, study by Mandi et al. showing that P-gp expression in the brain capillary endothelial cells is not modulated by TNF, but its function can be suppressed TNF (77). Interestingly, Theron et al. reported that TNF-α induces expression of P-gp at RNA, but not protein level; however, activity of P-gp decreases by the cytokine (78). This is supported by Billiard et al. showing that TNF-α

induces P-gp RNA expression, but decreases P-gp activity (79). While IFN-γ induce P-gp expression both RNA and protein levels, but has no effect on P-gp activity (79). At BBB, Bauer et a. demonstrated that TNF-α does not change P-gp expression, but decrease activity of P-gp (80). In contrast, Yu et al. showed increases in P-gp expression and function caused by TNF (81).

8. P-glycoprotein and cerebral malaria

There is no report associating role of P-gp on pathophysiology of cerebral malaria. However, reports showing antimalarial drugs as substrates of P-gp, including mefloquine and artemisinin (49, 82). In addition, mefloquine has inhibitory effect on P-gp. (51). In contrast, artemisinin has an influence on the expression of P-gp in primary human hepatocytes and in the human intestinal cell line LS174T. (82).

One of mechanisms by which malaria parasite resists to antimalarial drugs is to reduce accumulation of anti-malarial drugs. Efflux proteins have been identified in malaria parasite, including Pfmdr proteins (83), which is protein similar to P-gp (mdr1). However, relationships between malaria and human P-gp are still not known.

III. Function and pathology of cerebellum, cortex, brain stem, hippocampus and straitum

1. Cerebellum

Cerebellum is a part of brain that functions in balance, timing coordinated, smooth movement of skeletal muscle. Some conditions such as stroke cause damage to cerebellum, resulting in nausea, dizziness, balance and coordination difficulties. Dysfunction of cerebellum causes ataxia, which a symptoms involving a lack of coordination.

2. Cerebral cortex

Cerebral cortex is involved in vision, hearing, memory function, learning, emotion, language, decision making, problem solving, planning and the reception and processing of sensory information from the body.

3. Brain stem

Brain stem is responsible for basic vital life functions such as breathing, heartbeat, and blood pressure. This part of brain is also involved in vision, hearing, eye and body, motor control and sensory analysis. When brain stem is affected by diseases, several clinical manifestations are present, including visual disturbances, pupil abnormalities, changes in sensation, muscle weakness, hearing problems, vertigo, swallowing and speech difficulty, voice change, and co-ordination problems

4. Hippocampus

Hippocampus is a part of the limbic system, which is important for learning, spatial navigation and memory. It also has role in converting short term to more permanent memory. Damage of hippocampus is observed at the early stage of Alzheimer's disease. Oxygen starvation (anoxia or hypoxia) and encephalitis also cause hippocampus damage.

5. Striatum

Striatum is responsible in planning, modulation of movement pathways and other cognitive processes involving executive function. Dysfunction of striatum is present in Parkinson's disease, Huntington's disease, choreas, choreoathetosis and dyskinesias.

IV. Specific Aims

Specific aims of this study is based on the hypothesis that P-gp play role in pathophysiology of cerebral malaria. It is interesting whether or not malaria infection or CM has an influence of P-gp expression on capillary endothelial cells at BBB in patients with CM. Therefore, this research has aims to analyze expression of P-gp in cerebral malaria patients, compared to normal-control and encephalopathy patient groups.

Chapter 2 Methods

2.1. Sample Collection

Brain tissues (cerebral cortex, cerebellum, brain stem, hippocampus, and striatum) were collected within 12 hours postmortem from seven subjects who were divided into three groups; group 1 patients who died by cerebral malaria (n=3), group 2 patients who die by encephalopathy with no *Plasmodium falciparum* infection (n=1) and group 3 patients who died by the other causes which has no complication in brain (n=3). Brain tissues were then snap-frozen in liquid nitrogen (-196°C) and stored in -80°C until further analysis. Biological and clinical data were recorded, including age, gender, laboratory results, coma score (Glasgow score), prescriptions and other clinical manifestations and complications.

2.2. Separation of brain-capillary endothelial cells

Brain capillaries were isolated according to method reported earlier (84, 85). One gram of brain-tissue samples were used for separation of brain capillaries. First, white matter was removed and tissue dissociation was done by putting tissues in mortar. Then, 0.5 mL of 10% FBS-containing DMEM was added and tissues were then slowly grounded about ten times by hands. The homogenates were then transferred to 15-mL centrifuge tubes, the mortar was rinsed twice with 10% FBS-containing DMEM, and the homogenates were centrifuge at 1,000g for 10 minutes at 4°C. After that, micro-vessels were separated from myelin by adding DMEM containing 25% BSA and 10% FBS, followed by centrifugation at 1,500g for 20 minutes at 4°C. The supernatant was removed and wall of the 15-mL tube was cleaned by using cotton bud. Next, capillary fragment was separated by suspended the pellet from the previous step with 10% FBS-containing DMEM. The mixture was then filtered through nylon mesh with a pore size of 180 μm and, then, with a pore size of 41 μm. The filtrate was the centrifuged at 12,000g for 45 minutes at 4°C. The pellet was the resuspended with 1 mL of PBS and the mixture was centrifuged at 12,000g for 20 minutes at 4°C. The resulting pellet was the kept at -80°C until further analysis.

To prepare samples for protein analysis, the pellet was mixed with lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 126 mM NaCl, 1% Triton x-100, 0.1% SDS, and protease-inhibitor cocktail containing leupeptin, aprotinin, benzamidine, PMSF and pepstatine)

2.3. Determination of protein concentration

Protein in samples was determined by using BCA assay (Pierce, USA). Working reagent was prepared by mixing BCA solution (50 parts) and $CuSO_4$ (1 part). Ten or twenty-five microliters of diluent (blank), standard (25, 125, 250, 500, 750, 1,000, 1,500 and 2,000 μg/mL) or samples was pipette into microtiter plate. Two-hundred microliters of working reagent was then added, and the mixture was mixed and then incubated at 37°C for 30 minutes. Absorbance was then determined at 570 nm. The concentrations of protein were the calculated from standard curve.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were prepared and mixed with 5X loading buffer (20 mM Tris, pH6.8, 10% glycerol, 2% SDS, 150mM dithiothreitol (DTT) and 0.025% bromophenol blue). The samples were then separated in polyacrylamide gel prepared according to the table 2.1.

Samples were heat at 37°C for 5-10 min and loaded in polyacrylamide gel. Then, gel was run in SDS-PAGE running buffer (50 mM Tris, 384 mM glycine and 0.2% SDS) at 150 V constant until reaching the edge of separating gel and then run at 120 V constant. Proteins in polyacrylamide gel were then transferred to nitrocellulose membrane in protein transfer buffer (25 mM Tris, 192 mM glycine, 0.037% SDS and 15% Methanol) for further analysis with western blotting technique. To check efficiency of protein transfer, gel were stained with Coomasie-blue stain (0.1% Coomasie blue R250, 20% methanol and 10% acetic acid) and de-staining with the buffer containing 20% methanol and 10% acetic acid.

Table 2.1 Ingredients for preparation of polyacrylamide gel

Reagent	Separating gel (mL)	Stacking gel (mL)
	8% gel	4% gel
Deionized water	4.7	6.1
30% acrylamide: 0.8% bis-acrylamide (N,N'-methylene-bis-acrylamide)	2.7	1.3
Gel buffer - 1.5 M Tris, pH 8.8 for separating gel - 0.5 M Tris, pH 6.8 for stacking gel	2.5	2.5
10% SDS	0.1	0.1
10% Ammonium persulfate	0.1	0.1
TEMED (N,N, N', N'-tetramethylethylenediamine)	5 μL	10 μL

2.5. Western blotting

Nitrocellulose membrane was washed one time for 10 minutes in TBS (Tris buffer saline; 25 mM Tris, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20. The membrane was the blocked with 5% BSA-containing TTBS for 1 hour, and washed three times in TTBS for 10 minutes each. Primary antibody against P-glycoprotein (F4, Sigma-Aldrich, St. Louis, Missouri, USA) or actin (Zymed Lboratories Inc., South San Fancisco, CA, USA) was diluted in 3% BSA-containing TTBS and incubated with the membrane overnight at $4^{\circ}C$. The membrane was then washed three times in TTBS for 10 minutes each. Secondary antibody (HRP-conjugated anti-mouse IgG antibody, Pierce, Rockford, IL, USA) was diluted (1:5,000) in 3% BSA-containing TTBS and incubated with the membrane for 30 minutes. The membrane was then washed for three times in TTBS for 10 minutes each. Substrate was finally added on the membrane and incubated for 5 minutes. The membrane was the exposed to film for chemiluminescent signals.

2.6. mRNA Isolation

Isolation of total RNA was done by using RNeasy Lipid Tissue Kit (Qiagen, USA). Brain tissues (100 mg) were disrupted in QIAzol lysis reagent and stored at room temperature for 5 minutes. Chloroform (200 μL) was added to the lysates and mix vigorously for 15 seconds. The mixtures were then centrifuged at 12,000 xg for 15 minutes at $4^{\circ}C$. The top colorless part was transferred to a new tube, 600 μL of ethanol were added and the mixture was mix with vortex. Immediately, 700 μL of the mixture were transferred to RNeasy mini spin column placed in 2 mL collection tube, the collection tube was centrifuged at $\geq$ 8,000 xg for 15 seconds at room temperature, the flow-through was discarded. The previous steps were repeated by adding another 700 μL of the mixture to the same RNeasy mini spin column placed in 2 mL collection tube, centrifugating at $\geq$ 8,000 xg for 15 seconds at room temperature and discarding the flow-through. Buffer RW1 (700 μL) was then applied to the RNeasy mini spin column, subjected to centrifugation at $\geq$ 8,000 xg for 15 seconds. The column was then moved to a new collection tube and buffer RPE (500 μL) was added into the column, which was then centrifuged at $\geq$ 8,000 xg for 2 minutes. The column was then moved to a new collection tube, centrifuged with maximal speed for 1 minute. Then, RNA was eluted by moving the column to new 1.5 mL collection tube, adding 30-50 μL of RNase-free water to the column and centrifugation at $\geq$ 8,000 xg for 1 minute. Repeat the elution step and collected the flow-through for further steps.

2.7. Reverse transcription and Real-time PCR

Conversion of RNA to cDNA was done by using Omniscript Reverse Transcription kit (Qiagen, USA). First, the master mix was prepared by mixing oligo dT, dNTP mix, Omniscript reverse transcriptase, an appropriate amount of RNase-free water and 10X buffer RT. Template RNA (50 ng - 2 μg) was added to the master mix. The mixture was incubated at 37°C for 60 minutes. The resulting product was then transferred to ice and subjected to further step or storage at -80°C.

To measure amount of cDNA level, cDNA was subjected to real-time polymerase chain reaction (PCR). Primer and cDNA was added to the Quanti Tect SYBR green PCR master mix and subjected to PCR step by processing in the real-time PCR apparatus by using conditions as following, initial activation step (95°C, 15 minutes), 40 cycles of three-step cycling {denaturation (94°C, 15 seconds), annealing (55°C, 30 seconds), extension (72°C, 30 seconds). The resulting data was then acquired and analyzed.

2.8. Measurement of TNF-α by ELISA

Cytokines were extract from brain tissues by using previously reported method {Rosengren, 2003 #84; Matalka, 2005 #83}. Briefly, Ice-cold extracting buffer (PBS, containing 0.01% Triton X-100) was added to tissues (50 μL per 10 mg tissue), tissues were homogenized by hand on ice, incubated on ice for 60 minutes and centrifuged at 20,000 xg for 10 minutes at 4°C. The supernatant was the collected and stored at -80°C until further analysis.

TNF-α level was determined by using the Quantikine ELISA kit (R&D Systems Inc., USA). First, the kit was evaluated whether it can be use appropriately with the extraction-buffer matrix by “spike and recovery immunoassay sample validation” protocol provided by the supplier. First, “neat” (no addition of TNF-α), “spike”, “control” and “PBS” were as in Table 2.1.

Then, 50 μL of assay diluents were applied in pre-coated 96-well plate, and 200 μL of standard (1,000, 500, 250, 125, 62.5, 31.25, 15.6 pg/mL), diluted catlibrator diluents, sample (“neat”, “spike”, “control” and “PBS”) as well as diluted samples (1:2, 1:4 and 1:8) were loaded in to 96-well plate (Table 2.2.).

The mixtures were then incubated at room temperature for 2 hours, and the liquids were aspired. Washing with wash buffer (400 μL/wash) was done 4 times. The plate was inverted and excess liquid was blotted. Conjugate (200 μL) was then added in each well. Incubation of 1 hour at room temperature was done. The liquids were aspired, and plate was washed with wash buffer (400 μL/wash) 4 times. The plate was inverted and excess liquid was blotted. Development was done by adding 200 μL of substrate solution and incubating at room temperature for 20 minutes with protection from light. Fifty microliters of stop solution were then added. The color would change from blue to yellow. The absorbance at wavelength of 450 nm was measured within 30 minutes with the reference absorbance at wavelength of 570 nm. Concentration of TNF-α was calculated from standard curve, and percent recovery was calculated from the following formula;

$$\% \text{ Recovery} = \frac{\text{Recovered concentration}}{\text{Added concentration}} \times 100$$

$$\text{Added Concentration} = \text{Standard concentration} \times \frac{\mu\text{L standard}}{\mu\text{L standard} + \mu\text{L baseline sample}}$$

$$\text{Added Concentration} = 10{,}000 \text{ pg/mL} \times \frac{20\ \mu\text{L}}{20\ \mu\text{L} + 980\ \mu\text{L}}$$

$$= 200 \text{ pg/mL}$$

Correlation between expected concentrations of TNF-α and measured concentrations of TNF-α was assessed. Added concentrations were 200, 100, 50 and 25 pg/mL for sample ("spike", "control" and "PBS"), 1:2, 1:4 and 1:8 diluted samples, respectively.

Table 2.2. Preparations of “neat”, “spike”, “control” and “PBS” are shown

	Extracting buffer	**Tissue extract**	**Diluted calibrator diluent**	**Stock standard (10,000 pg/mL)**	**Other**
Neat	-	100 µL	900 µL	-	-
Spike	-	-	-	20 µL	980 µL “neat”
Control	98 µL	-	882 µL	20 µL	-
PBS	-	-	882 µL	20 µL	98 µL PBS

Table 2.3. Representation of plate layout used for spike and recovery immunoassay sample validation test

Column / Row	A	B	C
1	Standard 1,000 pg/mL	Neat	Control
2	Standard 500 pg/mL	1:2 Neat	1:2 Control
3	Standard 250 pg/mL	1:4 Neat	1:4 Control
4	Standard 125 pg/mL	1:8 Neat	1:8 Control
5	Standard 62.5 pg/mL	Spike	PBS
6	Standard 31.25 pg/mL	1:2 Spike	1:2 PBS
7	Standard 15.6 pg/mL	1:4 Spike	1:4 PBS
8	Diluted calibrator diluent	1:8 Spike	1:8 PBS

Chapter 3 Results

Cerebral malaria (CM) is a neurological complication found in some patients who are infected *P. falciparum*. Evidences show that the infection causes diffuse encephalopathy without entering of the malaria parasites into the brain parenchyma. Even though the breakdown of blood-brain barrier has been report, expression of P-glycoprotein (P-gp), which is an efflux protein, at the brain endothelial cells was not known. In the present study, expression of P-gp was assessed by using SDS-polyacrylamine gel electrophoresis and western blotting techniques. First, condition optimization was done by using HepG2 cells, which are hepatocyte cancer cells. It was found that boiling sample, a step in the conventional method, before loading into polyacrylamide gel was not suitable for P-gp blotting, giving no signals of P-gp. However, adjustment by warming samples at 37^{o}C resulted in P-gp signal in the blots. Using this condition, P-gp bands were observed as a 170 kD protein in both HepG2 cell lysate and brain samples (Figure 3.1a, b).

Brain-tissue samples (Cerebellum, cerebral cortex, brain stem, hippocampus and striatum) were collected from seven subjects who died because of cerebral malaria (subject no. 06, 17 and 20), encephalopathy with no *Plasmodium falciparum* infection (subject no. 19) and other causes with no brain complication in (subject no. 10, 11 and 12). Subject's data were recorded, including clinical manifestations, prescription and laboratory results (Table 3.1, Appendix I).

Brain-capillary endothelial cells were isolated and expression of P-gp was assessed. Even though equal amounts of protein in each sample were loaded, actin blotting was also done in parallel with P-pg blotting, as a loading control. We found that the levels of actin in each sample were not at the same level. Therefore, comparison of P-gp expression levels among samples was assessed by using band-density ratio between P-gp and actin band.

Expression of P-gp in brain-capillary endothelial cells was studied in five parts of brain tissues collected from seven subjects; cerebellum (Figure 3.2a), cerebral cortex (Figure 3.2b), brain stem (Figure 3.2c), hippocampus (Figure 3.2d) and striatum (Figure 3.2e). Band-density ratios between P-gp and actin bands were calculated and used as an value indicating changes in P-gp expression (Figure 3.3).

P-gp expression in brain-capillary endothelial cells isolated from subjects' cerebellum was assessed and comparisons among samples were done by using ratios between P-gp and

actin bands (Figure 3.2a, 3.3a). When compared to control patient no. 10 (the ratio of 1.68) and 11 (the ratio of 1.47), it was found that P-gp expression relatively decreased in the CM patient no. 06 (the ratio of 0.82) and 17 (the ratio of 0.79) and the encephalopathy patient no. 19 (the ratio of 0.79), while negligible change was found in CM patient no. 20 (the ratio of 1.46). Next, we analyzed P-gp levels in brain-capillary endothelial cells isolated from the cerebral cortex (Figure 3.2b, 3.3b). It was found that P-gp levels decreased in the CM patient no. 06 (the ratio of 0.81) and the encephalopathy patient no. 19 (the ratio of 0.57), compared to the control patient no. 10 and 11. There was a minimal change were observed in patient no. 17 and 20.

After that, P-gp levels in brain-capillary endothelial cells isolated from subjects' brain stem were examined (Figure 3.2c, 3.3c). When compared to control patient no. 10 (the ratio of 1.25) and 11 (the ratio of 1.79), it was found that P-gp in the CM patient no. 06 (the ratio of 0.74) and the encephalopathy patient no. 19 (the ratio of 0.89) decreased.

Subsequently, P-gp expression in brain-capillary endothelial cells isolated from subjects' hippocampus was assessed (Figure 3.2d, 3.3d). There were decreases in P-gp levels in CM patient no. 06 (the ratio of 0.91), while increases were observed in CM patient no. 20 (the ratio of 3.59) and encephalopathy no. 19 (the ratio of 2.81).

Finally, we analyzed P-gp expression levels in brain-capillary endothelial cells isolated from subjects' striatum (Figure 3.2e, 3.3e). It was found that P-gp levels decreased in brain-capillary endothelial cells isolated from CM patient no. 06 (0.98) and 17 (the ratio of 0.89) and encephalopathy patient no. 19 (the ratio of 0.94), compared to the control patient no. 10 (the ratio of 1.68). However, P-gp level in the patient no. 20 (the ratio of 1.25) was minimally changed. Changes in P-gp expression were summarized in Table 3.3.

Previous reports have shown correlation between expression of TNF-α and cerebral malaria. We, therefore, assessed TNF-α levels in brain samples by using ELISA technique. Before using to this technique to analyze TNF-α concentrations in brain samples, the ELISA kit was evaluated whether it is suitable for our system by doing "spike and recovery immunoassay sample validation" test. Cytokines were extracted by using extracting buffer, containing 0.1% Triton X-100, and TNF-α standard was added to tissue extract ("spike"), extraction buffer ("control"), and "PBS", with added concentration of 200 pg/mL. TNF-α concentrations in each sample were calculated from the standard curve (Figure 3.10). Amounts of TNF-α recovered

were 192.1, 179.9 and 113.3 for “spike”, “control” and “buffer”, respectively. Then percent recovery was calculated and results were 95.5%, 89.4% and 113%, respectively (Table 3.4). Acceptable range of percent recovery is 80 to 120. Therefore, this kit can be used with the extraction-buffer matrix. In addition, “spike”, “control” and “buffer” samples with added TNF-α standard were diluted 1:2. 1:4 and 1:8, and analyzed for TNF-α. Measured TNF-α concentrations were plotted against expected TNF-α concentrations. It was showed that relationships between expected and measured TNF-α concentrations are linear, representing linearity of the test. After that, sample extracts in brain samples were extracted using the extraction buffer and subjected to analysis of TNF-α by ELISA kit. TNF-α concentrations in samples were calculated from the standard curve. We could not detect TNF-α in our brain samples (data not shown).

Expression of TNF-α in brain samples were also assessed at mRNA levels and real-time, reverse-transcription polymerase-chain reaction (real-time, RT-PCR). However, PCR products were barely detected, even though high amounts of cDNA were used (1,000 ng, data not shown). In addition, expression of P-gp at mRNA levels was also limited by the quality of the cDNA.

Table 3.1. Subjects' data. Subject number, age, gender, admission to-death time and diagnosis were demonstrated.

Subject no.	Age	Gender	Diagnosis / Cause of death
10	32 y	M	Acute renal failure
11	26 y	M	Pesticide toxicity
12	27 y	M	Lung edema
06	35 y	M	Cerebral Malaria
17	6 m	F	Cerebral Malaria
20	40 y	M	Cerebral Malaria
19	24 y	F	Congenital heart failure with Anoxic encephalopathy

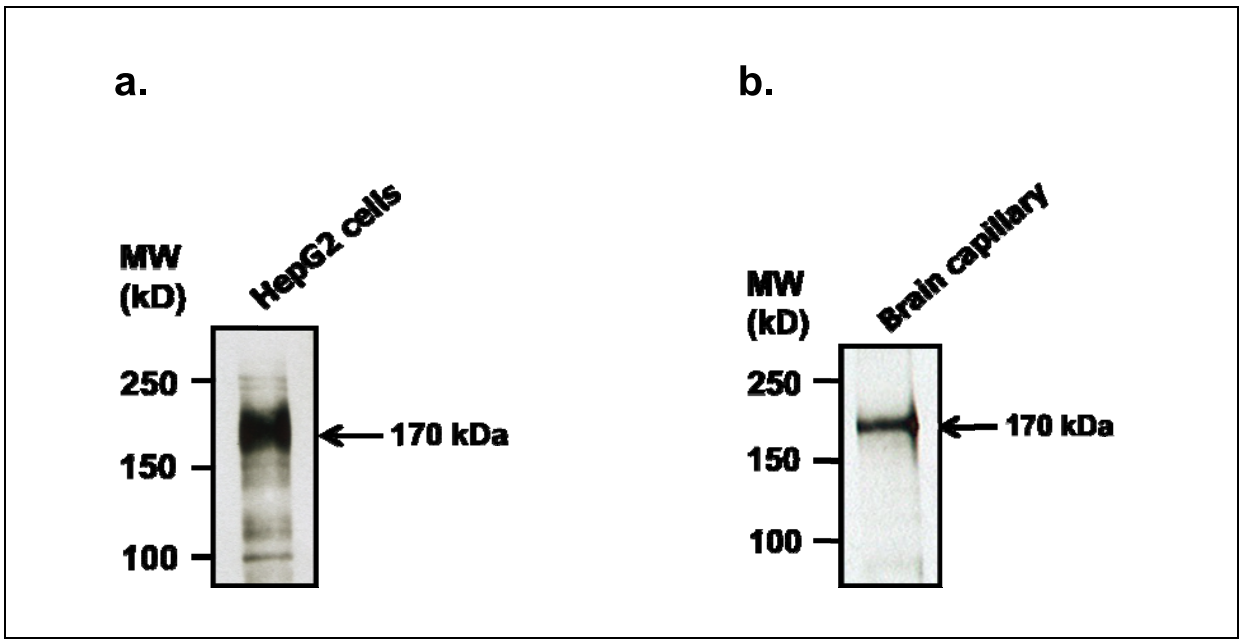


Figure 3.1. P-gp expression at protein level in HepG2 cells (a) and brain-capillary endothelial cells (b). HepG2 cells and brain-capillary endothelial cells were prepared and subjected to SDS-PAGE and western blotting with antibodies against P-glycoprotein.

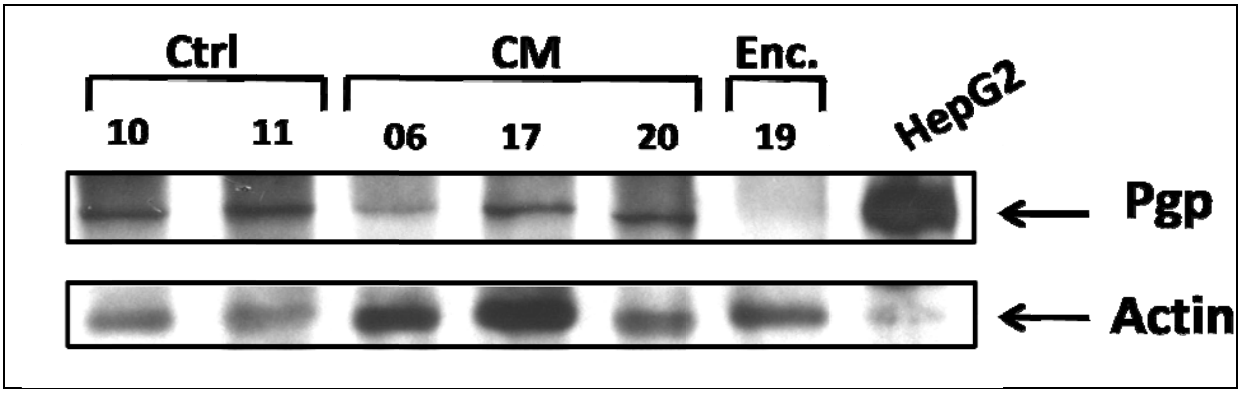


Figure 3.2 a

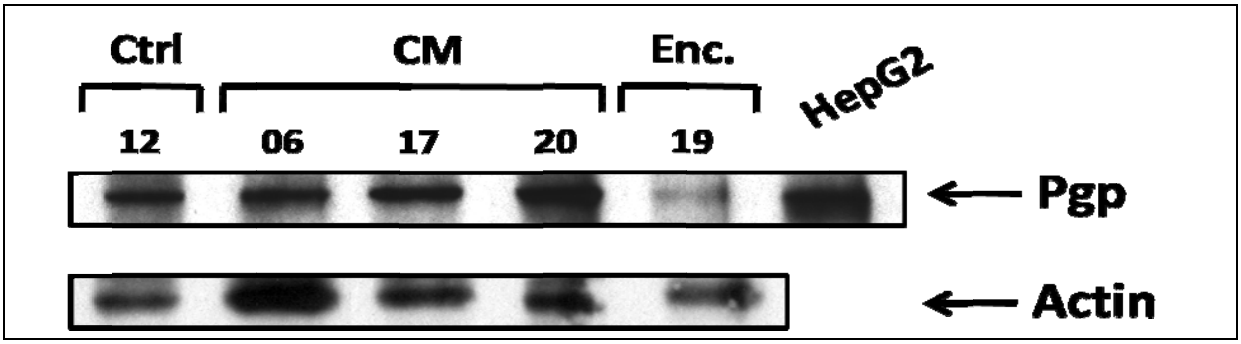


Figure 3.2b

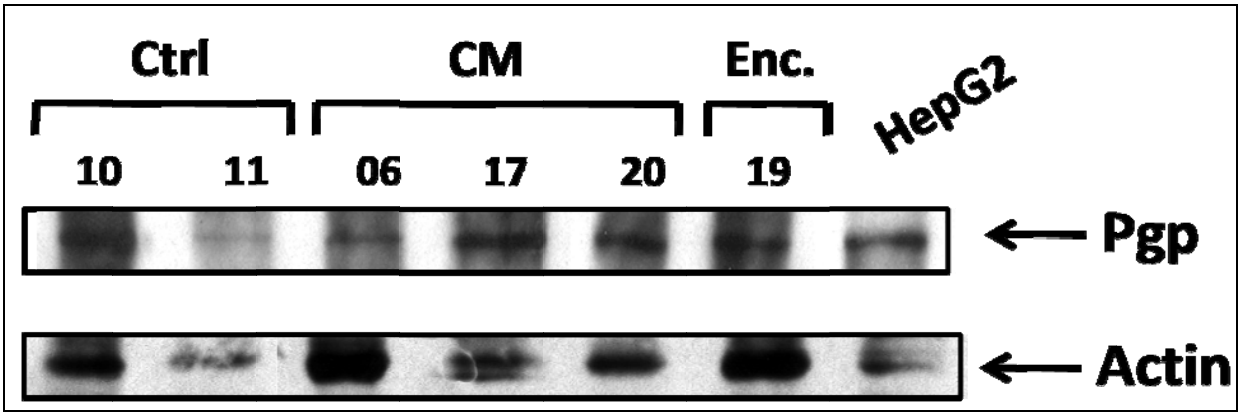


Figure 3.2c

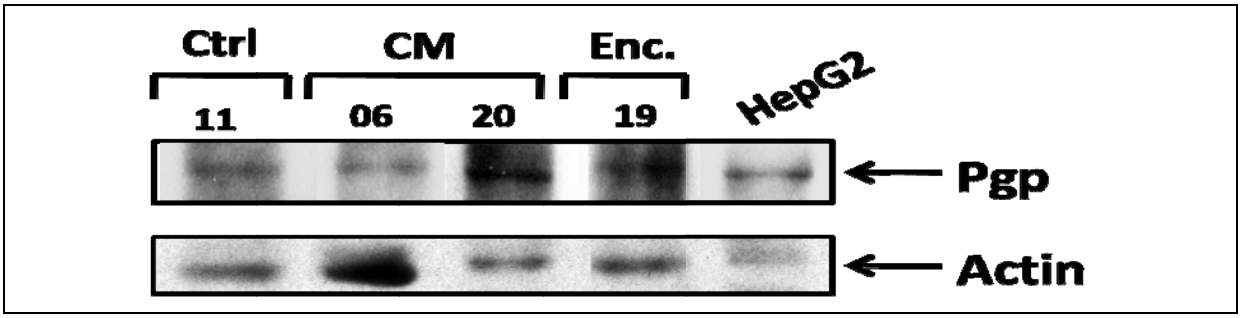


Figure 3.2d

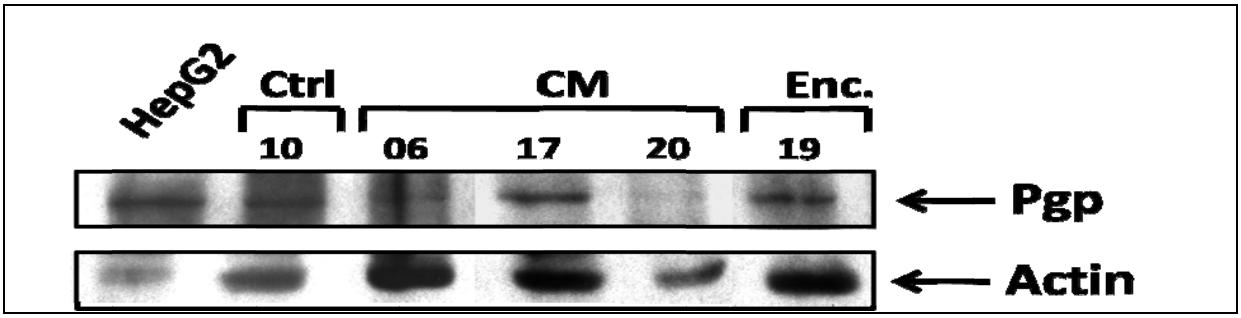


Figure 3.2e

Figure 3.2. Expression of P-glycoprotein (P-gp) in capillary endothelial cells isolated from brain tissues: cerebellum (a), cerebral cortex (b), brain stem (c), hippocampus () and striatum (e). Brain-capillary endothelial cells were prepared from cerebellum (a), cerebral cortex (b), brain stem (c), hippocampus () and striatum (e) collected from seven subjects: control (Ctrl, subject no. 10, 11 and 12), cerebral malaria (CM, subject no. 06, 17 and 20), encephalopathy Enc., subject no. 19). Expression of P-gp (upper panel) was assessed using SDS-PAGE and western blotting technique (see chapter 2). Actin (lower panel) was also blotted as loading control. HepG2 cell lysate was used as positive control. Subject numbers were labeled at the top of each lane.

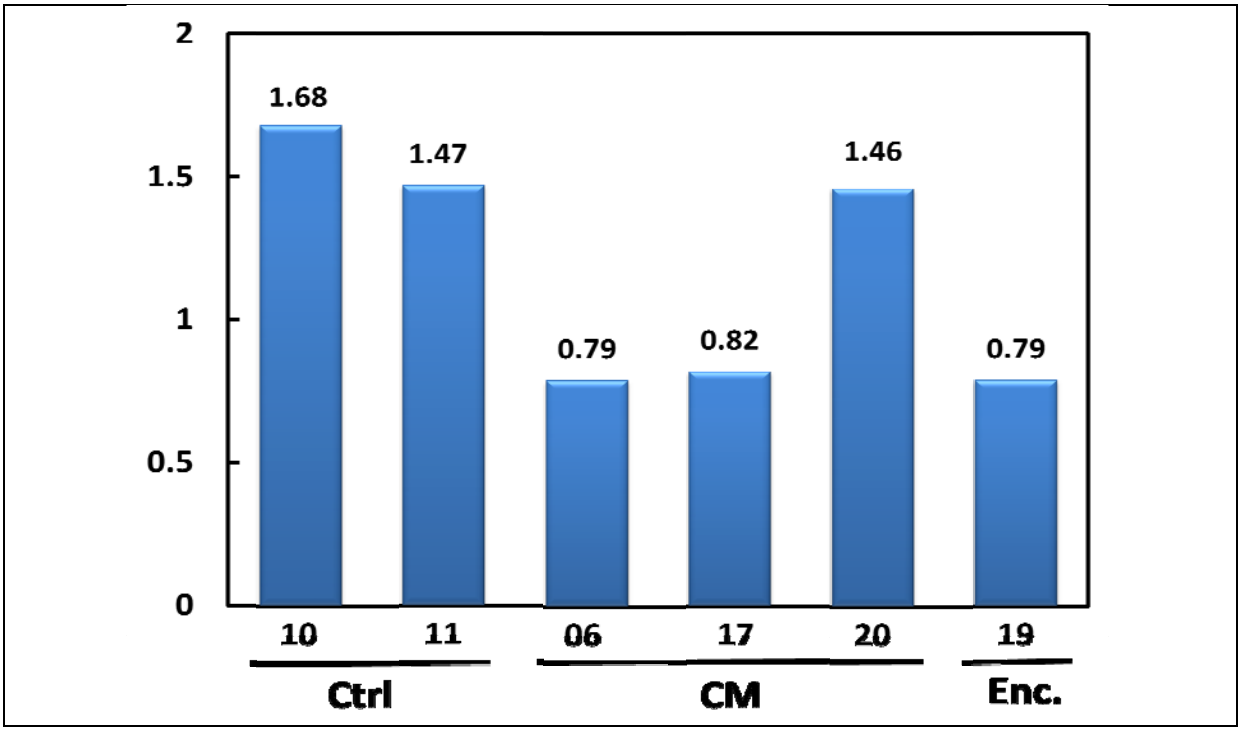


Figure 3.3a

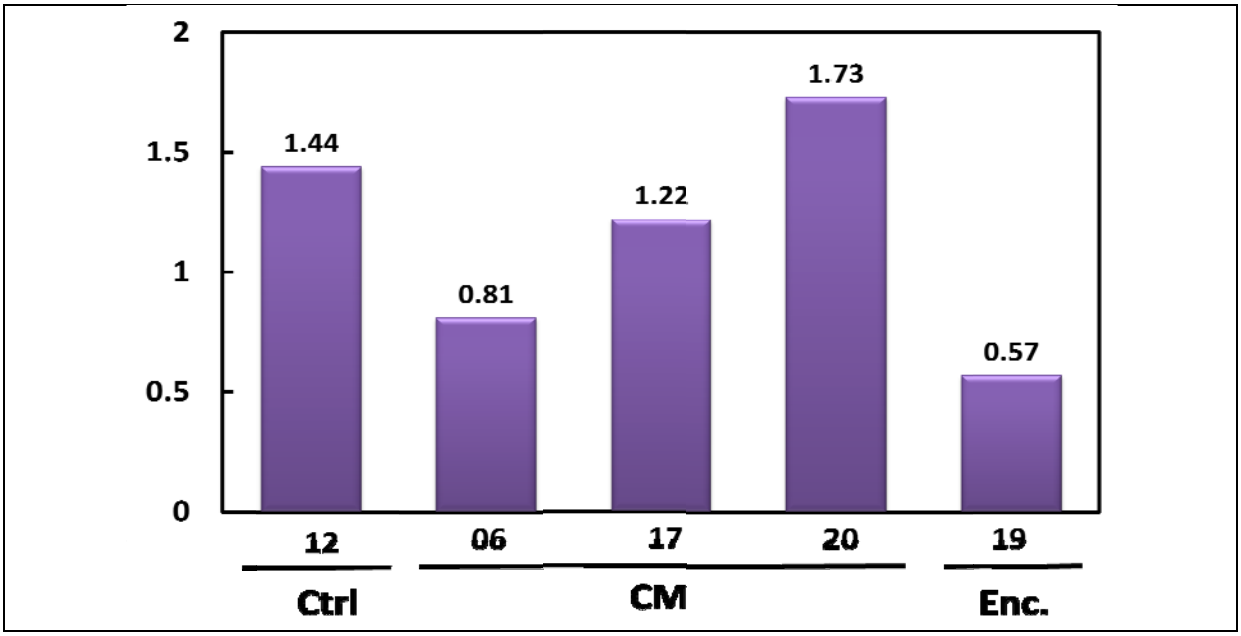
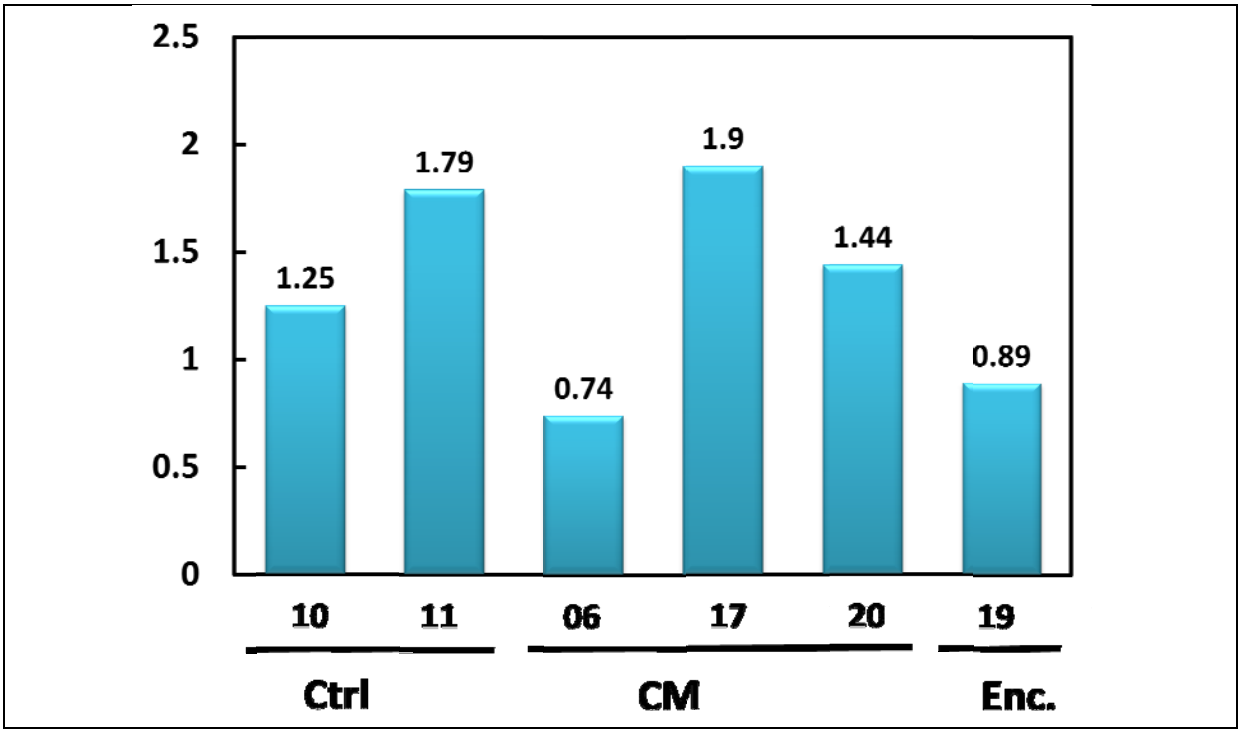


Figure 3.3b



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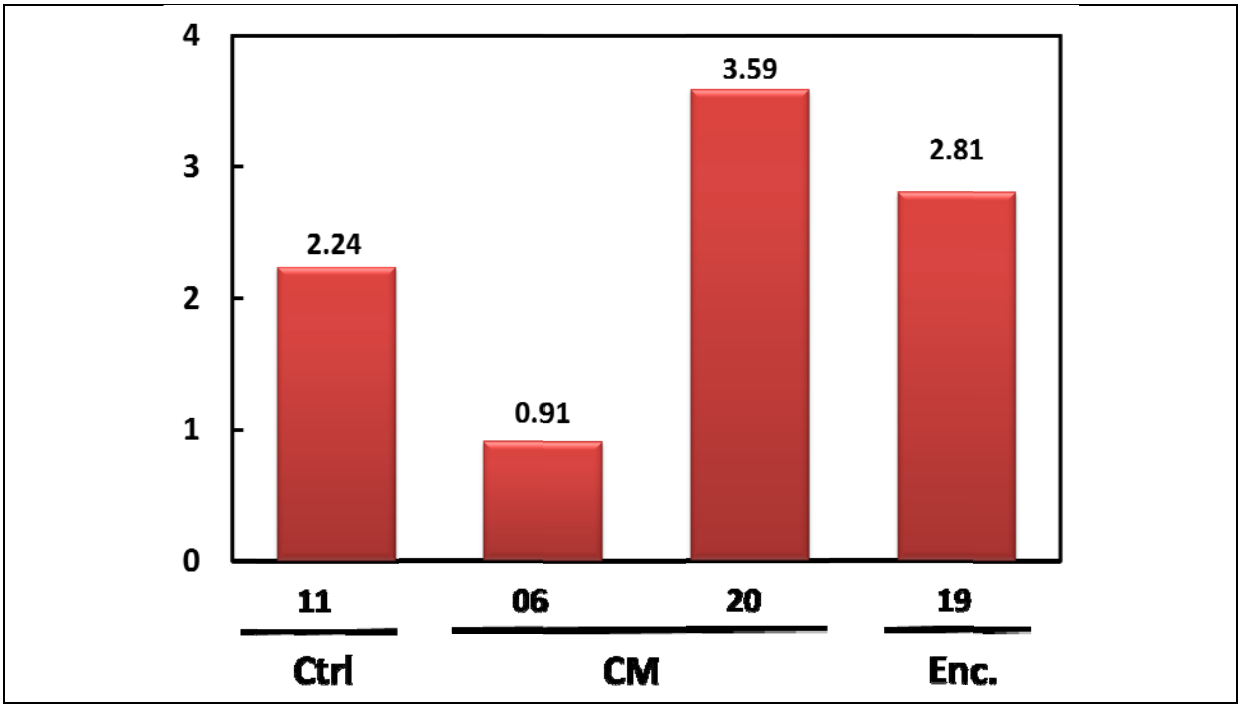


Figure 3.3d

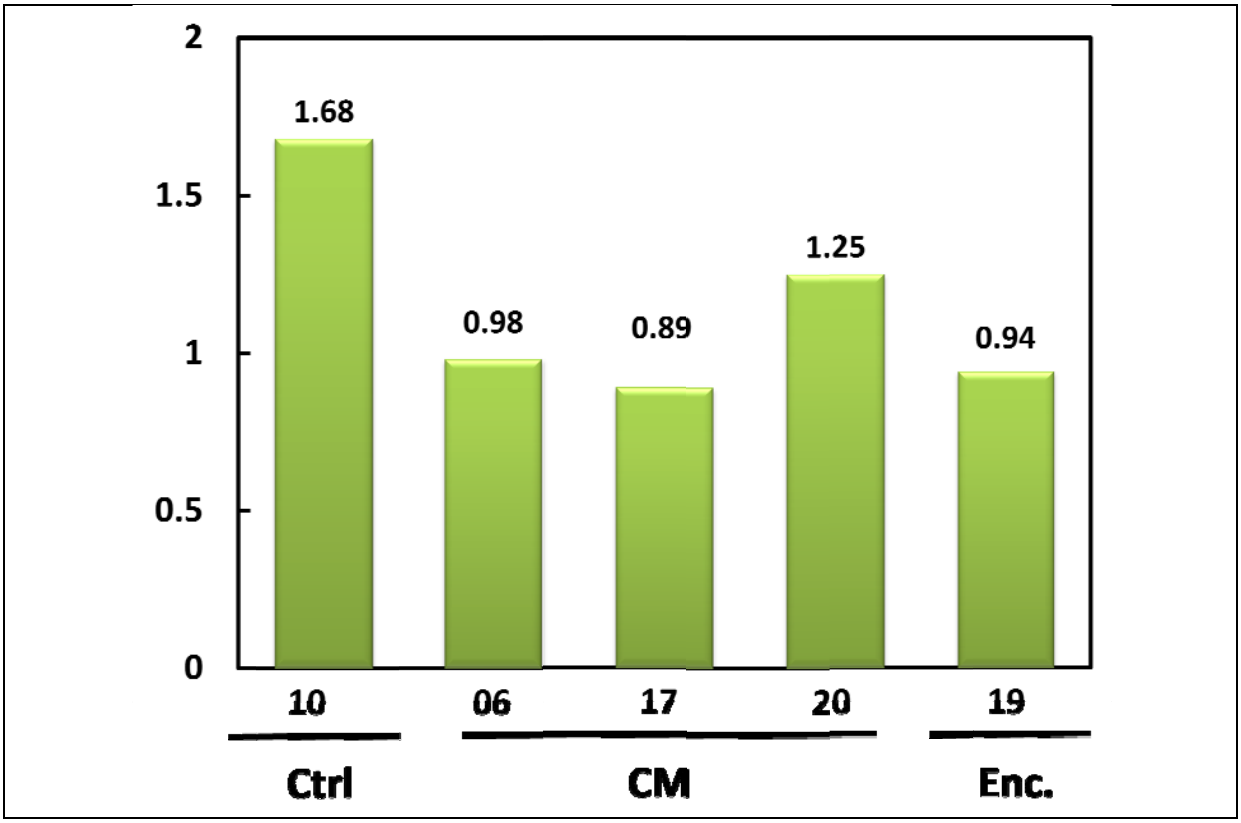


Figure 3.3e

Figure 3.3. Graphs represent the ratio of P-glycoprotein and actin-band densities. Expression of P-gp in capillary endothelial cells was assessed in cerebellum, cerebral cortex, brain stem, hippocampus and corpus striatum, collected from subject no. 06, 10, 11, 12, 17, 19 and 20, as mentioned in Figure 3.2. After film scanning, band densities and ratio between P-glycoprotein and actin band densities was calculated as shown.

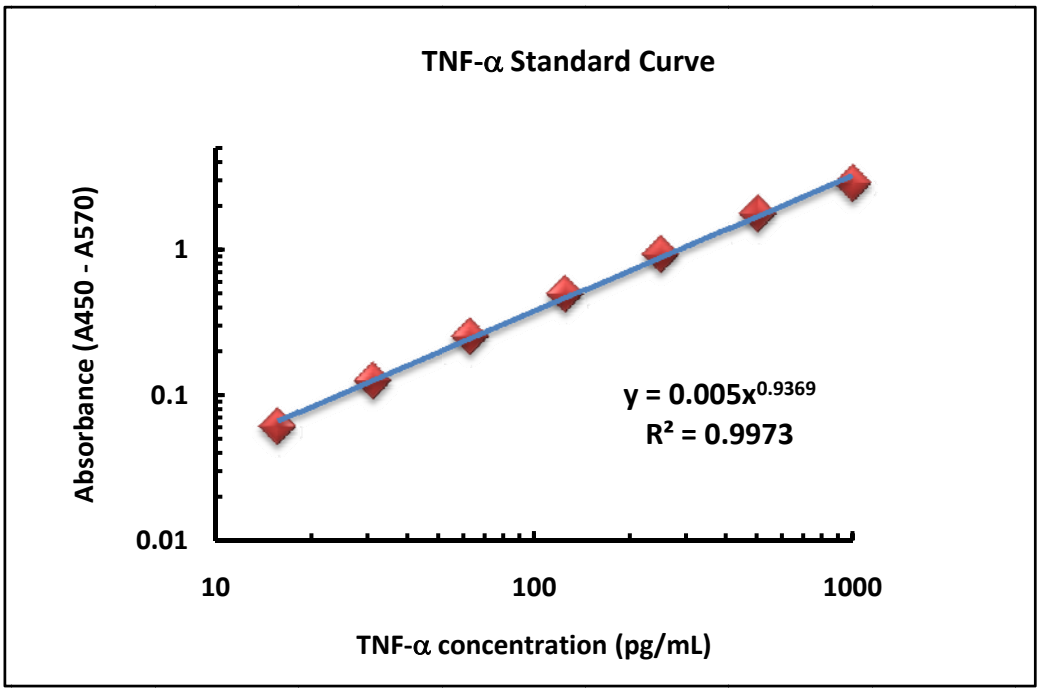
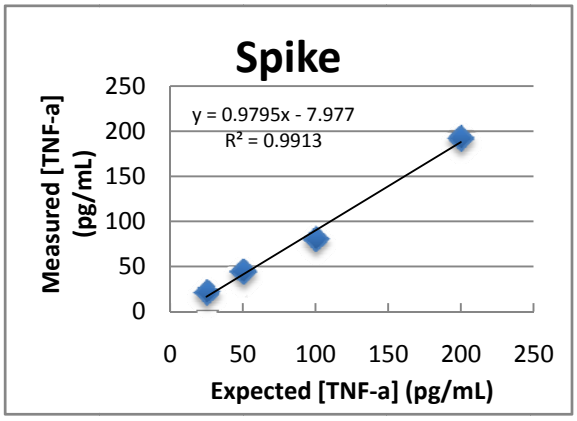
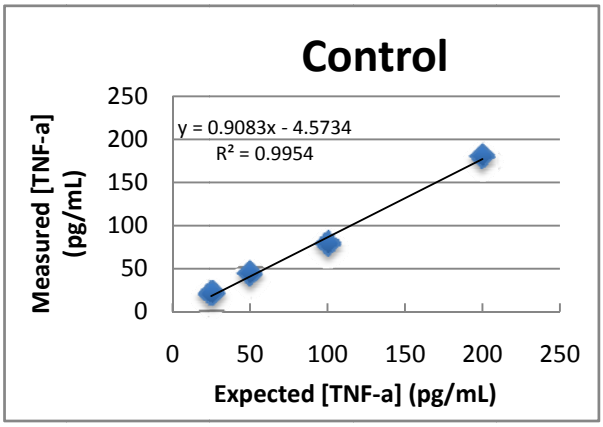


Figure 3.4. Standard curve of TNF-α determined by using Quantikine ELISA kit. TNF-α standards (1,000, 500, 250, 125, 62.5, 31.25, 15.6 pg/mL) were examined as described in Chapter 2. Absorbance at 450 (A450) and 570 (A570) nm was measured. The values of (A450 – A570) were plotted in log graph as Y-axis, and TNF-α concentrations were plotted as X-axis. Regression and R^2 value were calculated by using Microsoft Excel program.

a.



b.



c.

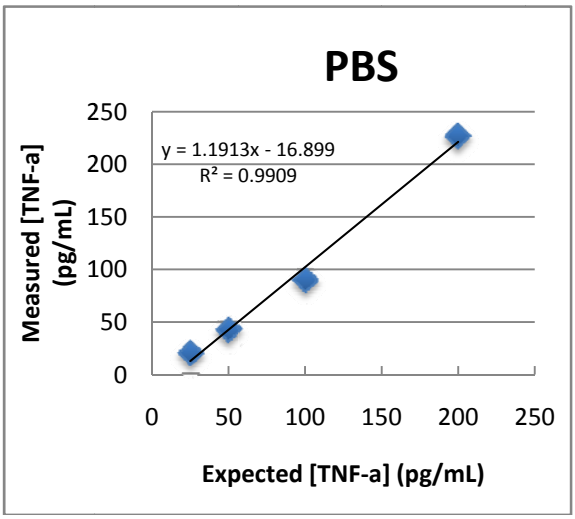


Figure 3.5. Graphs represent correlation between expected concentrations of TNF-α and measured concentrations of TNF-α. TNF-α levels were determined by using Quantikine ELISA kit, as described in chapter 2. Added concentrations in "spike" (a), "control" (b) and "PBS" (c) samples were set as X-axis, while measured concentrations were set as Y-axis. Linear regression was done and r^2 are shown.

Table 3.2. Spike and recovery immunoassay sample validation test

TNF-α standard was added to tissue extract, extraction buffer (PBS containing 0.1% Triton x-100) and PBS as described in Chapter 2. Levels of TNF-α were determined using R&D Quantikine ELISA kit. Percent recovery was calculated, as shown.

Matrix	Actual added concentration of TNF-α (pg/mL)	Measured concentration of TNF-α (pg/mL)	% recovery
Neat	200	192.09	95.5
Control	200	179.85	89.4
PBS	200	226.68	113.3

Chapter 4: Discussion and Conclusion

Cerebral malaria (CM) is a complex complication which may develop in individuals infected with *P. falciparum*. Since mortality and morbidity from CM continue to be reported, several studies have been done to investigate mechanisms of the disease with main goal to diminish such adverse effects of the disease, including neurological sequelae. In the present study, we showed that changes in P-gp expression at protein levels in samples (cerebellum, cortex, brain stem, hippocampus and striatum) were demonstrated in CM patients, as well as in patient with encephalopathy.

In the present study we studied P-gp expression in the brain-capillary-endothelial-cell samples isolated from seven subjects. It was found that P-gp expression in the samples isolated from subject number 06 decreased in all five parts of the brain, while other subjects revealed different pattern. The samples from subject number 17 showed a reduction only in cerebellum and striatum, while P-gp in the samples prepared from subject number 20 only reduced in striatum. Interestingly, subject number 19 (encephalitis) had a reduction of P-gp in 4 of 5 brain parts; cerebellum, cortex, brain stem, striatum. This represents inconsistency changes in P-gp expression affected by pathophysiological factors. These varying results may due to variations among individuals developing CM (86). The variations have been also seen by Brown et al. (17). They assessed expression of cytokines at both mRNA and protein levels in human CM. They showed that cortex, cerebellum and brain stem collected from CM patients showed expression of both TNF-α mRNA and protein; however, not all CM patients reveal positive results for cortex and brain stem. Only four of eight cortex samples and two of six brain stem samples produce positive results (detectable TNF-α product after RT-PCR). It is likely that there are several factors affecting host response to the infection. This is supported by studies revealing complexity of CM pathophysiology. Moreover, P-gp may be not the only one player inducing neurological consequence of CM.

At blood-brain barrier, a function of P-gp expressed on capillary endothelial cells includes transporting its substrates out of brain parenchyma. Therefore, decreases in P-gp can result in movement of molecules, including malaria antigen and toxin, from vascular space to

brain parenchyma. Consequently, these molecules cause activation of immune cells and releases of cytokines, leading to damage of neuron and subsequently, dysfunction of the affected brain area (87, 88). As suggested earlier by Jennings et al., they observed that cytokine production and infiltration of inflammatory cells in brain of CM mice (19). Our results showed the reduction of P-gp. This can imply role of P-gp in CM pathophysiology. However, reduction of P-gp was also observed in an encephalopathy subject who had no infection of *P. falciparum*. Therefore, changes in P-gp expression were not specific changes in CM, but common in encephalopathy. This is supported by the description of cerebral malaria as diffuse encephalopathy.

CM & cerebellum: Sequestration of malaria-infected red blood cells is more prominent in cerebellum than in cortex (89). It is possible that cerebellum is more favorable target of *P. falciparum* malaria. In addition, cerebellar ataxia, which is a symptom resulted from cerebellum damage, has been reported to be associated with *P. falciparum* malaria and CM (90). In addition, a study in India by Kochar et al. showed that most common neurological sequelae are psychosis and cerebellar ataxia (91). This represents damage of cerebellum is associated with CM, where neurological sequelae can develop. It is quite possible that P-gp is responsible to this adverse effect. Reduction of P-gp level and hence decreases in P-gp function result in increases in BBB permeability and passage of molecules from vascular to brain parenchyma. These molecules can be by malaria antigen or toxin, which induces release of cytokines by activation of leukemia (87, 88). The cytokines in turn modulate changes in CNS, and pathophysiology of CM.

P-gp in cerebral cortex: Studies have demonstrated pathological changes in cerebral cortex area. Krishnan et al. showed hemorrhagic infarction (cerebral vein thrombosis) in cerebral cortex in CM patients (92). Moreover, Clark et al. also reported alteration of cortex in CM mice (93). They showed that there are alteration of neurochemicals in cerebral cortex, including somatostatin, substance P and neuropeptide Y, reflecting functional abnormalities of brain function.

CM & Brain stem: Brain stem is the brain lesion which is mostly described to be affected in CM. Dysfunction of brain stem can lead to unconsciousness and coma. It is known

that brain stem neuropathy is observed in encephalopathies, including CM (94, 95) and leading to coma. Hypoxia is a cause of encephalopathy and found in CM. In human CM, abnormalities of brain-stem auditory evoked potentials have been observed. This change could result from petechial hemorrhages in brainstem (96). Neurological sequelae observed after recovered from CM also include dysfunction of brain stem (97). Therefore, brain stem defect is found in both CM and other encephalopathies. This knowledge supports our result showing similar decreases in P-gp in both CM and encephalopathy case.

CM & Hippocampus: In CM, pathological studies of hippocampus have been reported. Observation of petechial hemorrhage in CM is more frequently in hippocampus area. It was reported that hippocampus is damaged in CM-induced mice (98). Severe deficits in delayed memory and naming ability are responsible by hippocampal dysfunction. These disabilities have been reported as neurological sequelae of CM (99). Even though there is a good linkage between CM and hippocampal damage, we observed decreases in P-gp expression in one CM case, but not in the other CM case. For encephalopathy cases, only one CM sample showed decreases in P-gp expression. This result showed inconsistency among CM and encephalopathy. As mentioned, this may due to variations among individuals and other players involved in the pathogenesis. Nevertheless, hypoxia condition can be observed in both CM and encephalopathy. Therefore, changes in P-gp expression in hippocampus may be not definite for CM and encephalopathy, but hippocampus dysfunction may be caused hypoxia.

CM & Striatum: Even though not much has been reported about correlation between straitum and CM, it should be under consideration that mild parkinsonism because of striatal lesions may be seen in CM case (100). Necrosis of striatal lesions has been associated with several conditions, including severe metabolic acidosis, hypoxic encephalopathy and venous infarction. Since metabolic acidosis and hypoxic encephalopathy are seen in CM; therefore, it is quite possible that striatum damage also occurs. This is supported by study by Clark et al. (93), demonstrating changes in neurochemical, substance P, in striatum and possibly damage of neuron. Recently, it was reported that neuron damage is detected in CM mice (101) It is therefore likely that the changes play role on pathophysiology of CM. Our study showed that expression of P-gp was reduced in two cases of CM and two cases of encephalopathy cases, but we did not assess neurochemicals in brain samples. However, from previous study by

Clark et al. (93), damage of striatum is expected in CM. We observed a reduction of P-gp in all three CM and an encephalopathy subjects. Decreases in P-gp, therefore, could be a player because its reduction can increases BBB permeability and release of substances, leading to brain damage.

Our results demonstrated changes of P-gp expression in CM did not occur in all CM cases, except striatum. These may be because changes of P-gp expression are not necessary for CM pathophysiololgy and probably not directly related to cause of death.

Study of TNF-α in CM brain samples showed expression of TNF-α in cerebral cortex, cerebellum and brain stem. This cytokine can in turn activate vascular endothelial cells, resulting in up-regulation of ICAM-1. This adhesion molecule can mediate cytoadhesion of malaria-infected red blood cells which from rosetting and satellite with leukocytes or platelets. As mention in Chapter 1, P-gp expression can be modulated by several ways, including ROS and cytokines. Moreover, P-gp may be regulated at activity level, not expression level. Modulation of P-gp by ROS: When individuals are infected with malaria parasite, they are under stress (33, 34). Under such condition, ROS are produced, and P-gp expression can increase. This made infected individuals more resist to potentially toxic substances. Therefore, this is actually beneficial for host to defend themselves from neurotoxicity or other pathological consequences. In contrast, another study by Cai et al. revealed different results. They showed that ROS can also suppress expression of P-gp in K562/A02 cells (102). Another study in tumor spheroids by Wartenberg et al. showed that low levels of ROS (H_2O_2) decrease P-gp expression, while higher level of ROS upregulate P-gp expression. (103). These findings represent that ROS at different levels can produce different effects.

ROS have been reported to be associated with pathogenesis of CM. Their effects include direct damage of host tissues, including vascular endothelial cells. As a result, BBB is disrupted. Disruption of BBB has been implicated in pathophysiology of CM (104). In addition, ROS neutralization by superoxide dismutase supplement can protect endothelial cells from consequences causes by *P. falciparum* infection, including oxidative stress, apoptosis, up-regulation of adhesion molecule ICAM-1 and cytoadhesion of malaria-parasite infected red blood cells (105).

As described, interpretation of studies involving ROS have to be done with caution. Even though it is clear that hypoxia found in CM can lead to production of ROS and oxidative stress, it cannot be concluded that ROS would induce or suppress expression of P-gp in the context where individuals are infected with *P. falciparum* and developing CM.

Modulation of P-g by cytokines: P-gp expression can be also modulated by cytokines. Upregulation of cytokines during development of CM has been described. The most reported cytokine to be related to CM is TNF-α (16, 17, 19, 22, 23). It has been shown that TNF-α can inhibit both expression and activity of P-gp in human colon carcinoma cells (72). In contrast, TNF-α induces expression of P-gp mRNA in rat brain endothelial cells GPNT, with no change in protein levels (78). Interestingly, the cytokine induces an accumulation of P-gp substrate (vinblastine), reflecting inhibitory effect of TNF-α on P-gp activity. Hartz et al. also observed that TNF-α decreases transport of P-gp substrate at the BBB (76). The decreases in P-gp activity by TNF-α were also reported by other groups of researchers (79, 80). Therefore, modulation of P-gp can occur at both expression and activity levels by TNF-α.

In term of neurotoxicity of anti-malarial drugs, certain anti-malarial drugs can down-regulate P-gp and increase their neurotoxicity. Neurotoxicity by mefloquine has been reported. The mechanism is yet clear; however, it is known that mefloquine acts as both substrate and inhibitor of P-gp (51). Therefore, P-gp is potentially an important player in neurotoxicity pathway. In addition, quinine has been described as P-gp substrate (106) and mefloquine can increase accumulation of quinine in brain (106). This causes us to use a combination of mefloquine and quinine with caution. Moreover, potential neurotoxicity of the anti-malaria drugs artemisinin and its derivatives has been reported (94). Certain artemisinin derivative is potentially neurotoxic. However, Hien et al. did not observe neurotoxicity of artemether in individuals who were infected with *P. falciparum* and treated with artemether (107). Study in mouse model also showed a similar result (95). Therefore, it is still controversial, and more study is needed whether artemisinin is neurotoxic (108). In addition, study in rat treated with artemisinin showed that the drug is not being transported by P-gp at intestinal barrier (109). Therefore, artemisinin is not a substrate of P-gp and P-gp is not involved in its possible neurotoxic effect.

In the present study, We observed that P-gp expression in brain-capillary endothelial cells decreased in striatum collected from all three CM subjects, compared to control samples. However, the expression in cerebellum was reduced in two of three CM subjects and only one CM subject showed the reduction in brain stem, hippocampus and cerebral cortex. In the encephalopathy subject, P-gp expression was reduced in cerebellum, cerebral cortex, brain stem and striatum, but not in hippocampus. These results demonstrated that expression of P-gp can be modulated in CM and encephalopathy, but the modulation is dissimilar in different parts of brain.

As in the proposal, we aimed to correlated P-gp expression levels at both protein and mRNA levels, with any possible correlation with expression of cytokines (TNF-α). However, only the assessment of P-gp expression at protein level was done. This is because of limitation of quality and numbers of samples. However, previous studies have demonstrated the upregulation of TNF-α and resulting increases in ICAM-1 in CM (17, 22-25). Studies have revealed effects of the cytokines on P-gp expression and activity, but the conclusion is conflicting among different reports. Based on previous studies and our result, a model explaining roles of P-gp in CM could be depicted. It is quite possible that P-gp is modulated at both expression and activity levels, leading to pathogenesis of CM. P-gp can be modulated by both ROS, produced during hypoxia, and cytokines, especially TNF-α. Therefore, P-gp can be modulated by either expression or activity levels in CM where both ROS and TNF-α are produced in brain. These changes could lead to increases in permeability or disruption of BBB. Consequently, there is activation of immune cells, leading to damage of neuron and development of neurological complications. Therefore, further study remains for descriptive roles of P-gp in CM and encephalopathy. A defined set of experiments, such as an animal study, could be designed to further clarify roles of P-gp on pathophysiology of human CM an encephalopathy. Even though studying of CM pathophysiology is best done in human, but it is difficult to obtain enough amounts of good-quality postmortem brain samples from human subjects.

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Appendix

Appendix I Subject's data

1. General information

Patient no.	Age	Gender	Admit to death (h:m)	Clinical manifestations and complications	Prescription	Diagnosis
10	32 y	M	43:30	Convulsion, hypoglycemia, metabolic acidosis, apnea	Acetaminophen, Valium, Dopamine, Adrenaline,	Acute renal failure
11	26	M	N/A	Pesticide toxicity	N/A	Pesticide toxicity
12	27	M	N/A	Lung edema	N/A	Lung edema
06	35 y	M	39:26	Unconsciousness, coma (E1V1M1), hematuria, jaundice, pneumonia, anemia	Artesunate, Valium, Ranitidine, Lasix, Dexamethasone, Ranitidine, Acetaminophen, Thiamine, Cloxacillin, Dermicon, Dopamine, Pavulon, Adrenalin, Penicillin G	Cerebral Malaria
17	6 m	F	104:40	Fever, nausea, vomiting, unconsciousness, convulsion, bleeding, DIC, septic shock, severe anemia, metabolic acidosis, multi organ failure, renal failure	Cef-3, Ranithidine, Dopamine, Lasix, Domicum, Artesunate, Dobutamine, acetaminophen, Phenobarbital, Diazepam, Cloxacillin, Fortum, Fentanyl	Cerebral malaria
20	40 y	M	40:50	Unconsciousness, coma (E1V2M5 → E1VtM1), respiratory failure, septic shock, acute respiratory failure, renal failure, jaundice, acidosis	Dopamine, Cimet, Adrenaline, Artesunate, Ranithidine, Mefloquine, Primaquine, Lasix, Cef-3, Diazepam,	Cerebral malaria
19	24 y	F	37:55	Convulsion, confusion, sepsis, fever, bleeding	Valium, Cef-3, Lasix, Digoxin, Dilantin, Manital,	Congenital heart failure with Anoxic encephalopathy

2. Patient Laboratory results

Patient no. 10

Complete blood count		
Test	**Result (1st)**	
WBC	19000	
Neutrophil	78.5% (H)	
Lymphocyte	14.9% (L)	
Monocyte	1.8% (L)	
Eosinophil	3.4%	
Basophil	1.4% (H)	
RBC	4.85×10^6	
HCT	45.3%	
HGB	15.2 g/dl	
MCV	93.3 fl	
MCH	31.2 pg	
MCHC	33.5 g/dl	
MPV	9.1	
RDW	13.8%	
PLT	119000 (L)	
NRC	-	
Malaria	Not found	Not found

Others		
Test	**Result (1st)**	**Reference range**
Mg	1.6 mg%	1.5-2.5 mg%
Ca	6.4 mg%	8.4-10.4 mg%
PO_4	8.1 mg%	2.5-4.5 mg%
Uric acid	10.4 mg%	2.4-7 mg%
LDH	3786	114-240 U/L
CPK	18320	M < 175, W < 140 U/L
Glucose	-	70-110 mg%

Urine clearance		
Test	**Result (1st)**	**Reference range**
BUN	44 mg%	8-23 mg%
Creatinine	5.8 mg%	0.5-1.5 mg%
Liver function test		
Total Bili.	12.2 mg%	0-1 mg%
Direct Bili	3.72 mg%	0-0.35 mg%
AST	5140 U/L	5-40 U/L
ALT	2659 U/L	5-40 U/L
ALP	142 U/L	35-125 U/L
Total Protein	4.5 g%	6-8 g%
Albumin	2.5 g%	3.5-5 g%
Globulin	2 g%	2.5-3.5 g%
Electrolyte		
Na	132.1 mmol/l	135-148 mmol/l
K	5.18 mmol/l	3.7-5.3 mmol/l
Cl	89.8 mmol/l	98-106 mmol/l
tCO2	14 mmol/l	22-28 mmol/l
Blood Gases		
pH	7.247	7.35-7.45
pCO_2	21.7 mmHg	35-45 mmHg
pO_2	430 mmHg	75-100 mmHg
HCO_3	9.1 mmol/L	22-26 mmol/L
Coagulogram		
PT	-	
INR	-	
APTT	-	Control = 33.4

Urinalysis	
Test	**Result (1st)**
Color	Yellow
Sp.gr.	1.025
WBC	10-20 /HPF
RBC	10-20 /HPF
Squamous epi.	1-2
Albumin	Trace
Sugar	Neg
pH	6.0

Patient no. 06

Complete blood count		
Test	**Result (1^{st}/2^{nd})**	
WBC	6600 (R)	3500 (RL)
Neutrophil	85.8% (H)	20.5% (L)
Lymphocyte	5.4% (L)	76.8% (H)
Monocyte	8%	1.8% (L)
Eosinophil	0.1% (L)	0.1% (L)
Basophil	0.9%	0.8%
RBC	4.87 x 10^6	3.73 x 10^6
HCT	40.3 %	(L)
HGB	13.4 g/dl	30.6 % (L)
MCV	82.7 fl	9.9 g/dl (L)
MCH	27.5 pg	81.9 fl
MCHC	33.2 g/dl	26.7 pg
MPV	9.0 (R)	32.5 g/dl
RDW	15.8%	10.0 (R)
PLT	48000 (RL)	16.1%
NRC	-	69000 (RL)
		-
Malaria	Ring form of Pf - many	Ring form of Pf – many

Urine clearance		
Test	**Result (1^{st}/2^{nd}/3^{rd})**	**Reference range**
BUN	27 / 39 / 38	8-23 mg%
Creatinine	1.1 / 1.0 / 1.0	0.5-1.5 mg%
Liver function test		
Total Bili.	1.7 mg%	0-1 mg%
Direct Bili	0.41 mg%	0-0.35 mg%
AST	278 U/L	5-40 U/L
ALT	50 U/L	5-40 U/L
ALP	101 U/L	35-125 U/L
T. Protein	7.6 g%	6-8 g%
Albumin	3.8 g%	3.5-5 g%
Globulin	3.8 g%	2.5-3.5 g%
Electrolyte		
Na	128.1/132.8/129.7/130.8	135-148 mmol/l
K	4.2/4.4/4.6/5.4	3.7-5.3 mmol/l
Cl	102.8/106.9/107.6/107.9	98-106 mmol/l
tCO_2	19/17.3/17.6/15.8	22-28 mmol/l

Others		
Test	**Result (1^{st}/2^{nd})**	**Reference range**
Mg	-	1.5-2.5 mg%
Ca	8.8 mg%	8.4-10.4 mg%
PO_4	3.4 mg%	2.5-4.5 mg%
Uric acid	-	2.4-7 mg%
LDH	-	114-240 U/L
CPK	-	M < 175, W < 140 U/L
Glucose	-	70-110 mg%

Blood Gases		
pH	See table below	7.35-7.45
pCO_2		35-45 mmHg
pO_2		75-100 mmHg
HCO_3		22-26 mmol/L

Coagulogram		
PT	15.0	
INR	1.08	
APTT	35.0	Control = 33.4

Urinalysis		
Test	**Result (1st)**	**Result (2nd)**
Color	Yellow	Yellow
Sp.gr.	1.015	1.025
WBC	0-1 /HPF	1-2 /HPF
RBC	3-5 /HPF	20-30 /HPF
Squamous epi.	1-2	0-1
Albumin	2+	trace
Sugar	Neg	Neg
pH	6.0	6.0
Cast	Granular 2-3 /LPF	-
Amorphous	-	-
Bacteria	few	many

Blood Gases

Test	**Result (1st)**	**Result (2nd)**	**Result (3rd)**	**Result (4th)**	**Result (5th)**
pH	7.576	7.374	7.456	7.386	7.282
pCO_2	15.6 mmHg	30.1 mmHg	26.5 mmHg	26.7 mmHg	29.3 mmHg
pO_2	495.0 mmHg	98.3 mmHg	95 mmHg	83.2 mmHg	87.6 mmHg
HCO_3	14.5 mmol/L	17.1 mmol/L	18.4 mmol/L	15.7 mmol/L	13.4 mmol/L

Bacteria (specimen = pus)

Test	**Result**
Gram	1. gram + cocci in single, pair and short chain - many 2. gram + cocci in cluster - few

Patient no. 17

Complete blood count				**Urine clearance**			
Test	**Result ($1^{st}/2^{nd}/3^{rd}$)**			**Test**	**Result ($1^{st}/2^{nd}/3^{rd}$)**		**Reference range**
WBC	42700	15500	22400	BUN	10/39/77		8-23 mg%
Neutrophil	30%	75%	-	Creatinine	1.3/1.7/3.4		0.5-1.5 mg%
Lymphocyte	65% (H)	19%	-	**Liver function test**			
Monocyte	5%	6%	-	Total Bili.	-		0-1 mg%
Eosinophil	0%	0%	-	Direct Bili	-		0-0.35 mg%
Basophil	0%	0%	-	AST	-		5-40 U/L
RBC	0.69×10^6(L)	3.56×10^6 (L)	-	ALT	-		5-40 U/L
HCT	6.1 % (L)	29.2 % (L)	-	ALP	-		35-125 U/L
HGB	2.2 g/dl (L)	10.2 g/dl (L)	-	T-Protein	-		6-8 g%
MCV	88.1 fl	82 fl	8.9% (L)	Albumin	3.0 g%		3.5-5 g%
MCH	31.7 pg	28.8 pg	-	Globulin	-		2.5-3.5 g%
MCHC	35.9 g/dl	35.1 g/dl	-	**Electrolyte**			
MPV	9.0 (R)	-	-	Na	See table below		135-148 mmol/l
RDW	19.3 (H)	14.5	-	K			3.7-5.3 mmol/l
PLT	50000(RL)	14000	-	Cl			98-106 mmol/l
NRC	-	81/100wbc	-	tCO2			22-28 mmol/l
Malaria	Ring form + gametocyte of PF - many	Ring form + gametocyte of PF - modurate	Ring form + gametocyte of PF	**Blood Gases**			
				pH	7.342	7.362	7.35-7.45
				pCO_2	21.7	14	35-45 mmHg
				pO_2	58.8	464	75-100 mmHg
				HCO_3	11.4	-	22-26 mmol/L

Others		
Test	**Result (1^{st})**	**Reference range**
Mg	-	1.5-2.5 mg%
Ca	9.9 mg%	8.4-10.4 mg%
PO_4	-	2.5-4.5 mg%
Uric acid	-	2.4-7 mg%

Coagulogram		
PT	-	
INR	-	
APTT	-	Control = 33.4

Electrolyte

Test	Result (1st)	Result (2nd)	Result (3rd)	Result (4th)	Result (5th)
Na	139.8 mmol/l	147.0 mmol/l	144.1 mmol/l	141.3 mmol/l	133.7 mmol/l
K	4.80 mmol/l	4.24 mmol/l	3.59 mmol/l	3.99 mmol/l	3.58 mmol/l
Cl	98.5 mmol/l	100.1 mmol/l	97.9 mmol/l	98.1 mmol/l	82.7 mmol/l
tCO_2	< 5 mmol/l	8.8 mmol/l	16.1 mmol/l	19.0 mmol/l	19.1 mmol/l

Patient no. 20

Complete blood count			
Test	**Result (1^{st}/2^{nd})**		
WBC	15100 (H)	23500 (H)	17300 (RH)
Neutrophil	69.8%	85.6% (H)	72.9%
Lymphocyte	25.5%	11.7% (L)	23.1% (L)
Monocyte	3.5%	1.8% (L)	2.9% (L)
Eosinophil	1.1%	0.7%	1.1%
Basophil	0.1%	0.2%	0.0% (L)
RBC	2.80 x 10^6(L)	2.41 x 10^6(L)	2.94 x 10^6(L)
HCT	23.2 % (L)	20.1 % (L)	23.0 % (L)
HGB	8.2 g/dl (L)	6.8 g/dl (L)	7.9 g/dl (L)
MCV	82.8 fl	83.4 fl	78.2 fl
MCH	29.1 pg	28.1 pg	27 pg
MCHC	35.1 g/dl (H)	33.7 g/dl (H)	34.6 g/dl
MPV	8.2 (R)	-	-
RDW	16.2%	16.5%	15.7%
PLT	19000(RL)	9000 (RL)	24000 (RL)
NRC	-	-	-
Malaria	Ring form + gametocyte of PF - many	Ring form + gametocyte of PF - many	Ring form + gametocyte of PF - many

Urine clearance		
Test	**Result (1^{st}/2^{nd})**	**Reference range**
BUN	72/105	8-23 mg%
Creatinine	2.1/3.6	0.5-1.5 mg%
Liver function test		
Total Bili.	4.5 mg%	0-1 mg%
Direct Bili	1.57 mg%	0-0.35 mg%
AST	756 U/L	5-40 U/L
ALT	237 U/L	5-40 U/L
ALP	173 U/L	35-125 U/L
Total Protein	4.1 g%	6-8 g%
Albumin	2.1 g%	3.5-5 g%
Globulin	2.0 g%	2.5-3.5 g%
Electrolyte		
Na	See table below	135-148 mmol/l
K		3.7-5.3 mmol/l
Cl		98-106 mmol/l
tCO_2		22-28 mmol/l

Others		
Test	**Result**	**Reference range**
Mg		1.5-2.5 mg%
Ca		8.4-10.4 mg%
PO_4		2.5-4.5 mg%
Uric acid		2.4-7 mg%
LDH	-	114-240 U/L
CPK	-	M < 175, W < 140 U/L
Glucose		70-110 mg%

Blood Gases		
pH	7.301/7.328	7.35-7.45
pCO_2	28.9/35.1	35-45 mmHg
pO_2	27.7/341.6	75-100 mmHg
HCO_3	13.8/17.9	22-26 mmol/L
Coagulogram		
PT		
INR		
APTT		Control = 33.4

Urinalysis	
Test	**Result (1st)**
Color	Yellow
Sp.gr.	1.030
WBC	10-20 /HPF
RBC	30-50 /HPF
Squamous epi.	3-5
Albumin	trace
Sugar	Neg
pH	5.0
Cast	Granular 5-10 /LPF
Amorphous	4+

Electrolyte

Test	**Result (1st)**	**Result (2nd)**	**Result (3rd)**
Na	111.9 mmol/l	142.4 mmol/l	140.8 mmol/l
K	5.78 mmol/l	7.13 mmol/l	3.79 mmol/l
Cl	85.7 mmol/l	107.8 mmol/l	102.2 mmol/l
tCO_2	5.0 mmol/l	5.5 mmol/l	23.1 mmol/l

Patient no. 19

Complete blood count	
Test	**Result (1st)**
WBC	12800
Neutrophil	84.4% (H)
Lymphocyte	9% (L)
Monocyte	6%
Eosinophil	0.1%
Basophil	0.5%
RBC	4.31 x 10^6
HCT	(L)
HGB	38 % (L)
MCV	13.1 g/dl (L)
MCH	88.2 fl
MCHC	30.3 pg
MPV	34.4 g/dl
RDW	8.6
PLT	12.3%
NRC	353000

Malaria	Not found	

Others		
Test	**Result (1st)**	**Reference range**
Mg	-	1.5-2.5 mg%
Ca	-	8.4-10.4 mg%
PO_4	-	2.5-4.5 mg%
Uric acid	-	2.4-7 mg%
LDH	-	114-240 U/L
CPK	-	M < 175, W < 140 U/L
Glucose	111 mg%	70-110 mg%

Urine clearance		
Test	**Result (1st)**	**Reference range**
BUN	23 mg%	8-23 mg%
Creatinine	1.1 mg%	0.5-1.5 mg%
Liver function test		
Total Bili.	0.6 mg%	0-1 mg%
Direct Bili	0.16 mg%	0-0.35 mg%
AST	55 U/L	5-40 U/L
ALT	60 U/L	5-40 U/L
ALP	67 U/L	35-125 U/L
Total Protein	7.4 g%	6-8 g%
Albumin	4.3 g%	3.5-5 g%
Globulin	3.1 g%	2.5-3.5 g%
Electrolyte		
Na	144.3 mmol/l	135-148 mmol/l
K	4.55 mmol/l	3.7-5.3 mmol/l
Cl	107.4 mmol/l	98-106 mmol/l
tCO2	18.4 mmol/l	22-28 mmol/l

Blood Gases		
pH	-	7.35-7.45
pCO_2	-	35-45 mmHg
pO_2	-	75-100 mmHg
HCO_3	-	22-26 mmol/L
Coagulogram		
PT	-	
INR	-	
APTT	-	

Urinalysis	
Test	**Result (1st)**
Color	Yellow
Sp.gr.	1.025
WBC	10-20 /HPF
RBC	1-2 /HPF
Squamous epi.	3-5
Albumin	2+
Sugar	Neg
pH	6.0
Cast	Granular 5-10 /LPF
Amorphous	2+

Bacteria (specimen = sputum)

Test	**Result**
Gram Stain	Gram + cocci in single, pair, chain - moderate
AFB	Not seen, WBC 2+

Appendix II Description of Drugs

Drug name	Description
Quinine*	- Anti-malarial drug with mild antipyretic and analgesic effect - An alkaloid derived from the bark of the cinchona tree.
Primaquine*	- Anti-malarial drug - An aminoquinoline - Producing a radical cure and prevent relapse of *P. vivax* and *P. ovale* malarias - Preventing transmission of *P. falciparum* malaria - Adverse effects include anemias and GI disturbances
Mefloquine*	- Anti-malarial drug - Imteracting with phospholipid - Very effective against plasmodium falciparum with very few side effects
Artesunate*	- Anti-malarial drug
Atropine	- An alkaloid with inhibitory effect on parasympathetic function - Competitive inhibitor of acetylcholine at muscarinic receptor - Reducing secretion of saliva, mucous, secretion in esophagus - Reduce contraction of esophagus and intestinal movement
Cimet (Cimetidine)	- Anti-histamine drug by inhibition of histamine binding to histamine H2 receptors - Inhibiting gastric acid, pepsin and gastrins secretion - Blocking the activity of cytochrome P-450
Ranitidine	- A non-imidazole blocker of histamine H2 receptors - Inhibiting gastric secretion (receptors). - Used to treat gastrointestinal ulcers.
Adrenaline (Epinephrine)	- Active sympathomimetic hormone from the adrenal medulla - Stimulating both the alpha- and beta- adrenergic systems - Causing systemic vasoconstriction and gastrointestinal relaxation, stimulates the heart, and dilates bronchi and cerebral vessels. - used in asthma and cardiac failure and to delay absorption of local anesthetics

Drug name	Description
Digoxin	- A cardiotonic glycoside with positive inotropic and negative chronotropic activity - Used to control ventricular rate in atrial fibrillation and in the management of congestive heart failure with atrial fibrillation.
Manital	- Used to reduce skull pressure which is increased because of hyper-ventilation
Domicum (Midazolam)	- Sleeping pill in Benzodiazepine group - Causing anterograde amnesia
Phenobarbital	- Anticonvulsant
Dilantin	- Anticonvulsant
Valium, Diazepam	- A benzodiazepine with anticonvulsant, anxiolytic, sedative, muscle relaxant and amnesic properties with a long duration of action - Stimulating gamma-aminobutyric acid activity - Used in the treatment of severe anxiety disorders
Pavulon	- A neuromuscular blocking steroid with a competitive nicotinic antagonist effect
Dobutamine (Dobutrex)	- A beta-2 agonist catecholamine with cardiac stimulant action without evoking vasoconstriction or tachycardia.
Lasix	- diuretics - Stimulating excretion of water and mineral
Dopamine	- Catecholamine neurotransmitters in the brain - A major transmitter in the extrapyramidal system of the brain, and important in regulating movement
Fentanyl	- A mu-opioid agonist - A potent narcotic analgesic, abuse of which leads to habituation or addiction. - Used as an adjunct to general anesthetics, and as an anesthetic for induction and maintenance
Somatotulin	- Estrogen

Drug name	Description
Cef-3 (Cefteiaxone, Cefotaxime, Ceftazidime, Fortum)	- broad-spectrum antibiotic (third-generation cephalosporins) - Used especially for Pseudomonas and other gram-negative infections in debilitated patients
PGS (Pennicillin G Sodium, Cloxacillin, Dermicon, Metronidazole, Amiodazole)	- Antibiotic, derivative of oxacillin
Chloramphenical	- Broad-spectrum antibiotic - Acting by interfering with bacterial protein synthesis and is mainly bacteriostatic
Dexamethasone	- An anti-inflammatory 9-fluoro-glucocorticoid
Sandostatin	- Octreotide, which is a long-acting octapeptide with pharmacologic properties - mimicking natural hormone somatostatin action
Lactulose	- A synthetic disaccharide used in the treatment of constipation and hepatic encephalopathy. - Used in the diagnosis of gastrointestinal disorders

Note: *: Antimalaria drug

Malaria Infection	Anti-malarial drug
P. falciparum	- First line drug: Mefloquine and primaquine combination - Second line drug: quinine, tetracycline and primaquine combinations - Third line drug: artesunate or artemether and primaquine combinations
P. vivax and *P. ovale*	Chloroquine and primaquine combinations
P. malariae	Chloroquine

Appendix III OUTPUT

*** The manuscript is in preparation to submit.

Appendix IV บทความสำหรับเผยแพร่

P-glycoprotein (P-gp) เป็นโปรตีนชนิด efflux protein ซึ่งพบอยู่บนเซลล์หลายชนิด รวมถึงเซลล์บุผนังหลอดเลือดที่ blood-brain barrier (BBB) หน้าที่ของ P-gp ที่ BBB ได้แก่การป้องกันไม่ให้โมเลกุลที่อาจทำให้เกิดพิษเข้าสู่เนื้อสมอง มาลาเรียขึ้นสมองเป็นภาวะแทรกซ้อนในสมองส่วนกลางที่พบในมาลาเรียขั้นรุนแรง ภาวะนี้เกิดจากการติดเชื้อมาลาเรียชนิด *Plasmodium falciparum* ในภาวะมาลาเรียขึ้นสมอง พบว่ามีการเปลี่ยนแปลงเกิดขึ้นหลายอย่างในสมองส่วนกลางทำให้เกิดอาการทางประสาท การเสียชีวิตและอาจรวมถึงผลกระทบต่อระบบประสาทที่อาจพบหลังจากการหายของโรค ในศึกษานี้ มีการประเมินการแสดงออกของ P-gp ระดับโปรตีนในตัวอย่างเนื้อเยื่อสมองที่เก็บหลังจากการเสียชีวิต โดยมีการเก็บสมองห้าส่วน (cerebellum, cerebral cortex, brain stem, hippocampus และ striatum) จากผู้เข้าร่วมการวิจัย จำนวน 7 ราย ซึ่งแบ่งเป็น 3 กลุ่ม คือ 1) ผู้ที่เป็นมาลาเรียขึ้นสมอง (n=3), 2) ผู้ที่เป็น encephalopathy โดยไม่มีการติดเชื้อ *P. falciparum* (n=1) และ 3) ผู้ที่ไม่มีการแทรกซ้อนในสมอง (กลุ่มควบคุม, n=3) เซลล์บุผนังหลอดเลือดฝอยในสมองถูกแยกออกมาเพื่อการวิเคราะห์ระดับของการแสดงออกของ P-gp โดยใช้วิธี SDS-PAGE และเทคนิค western blotting เราพบว่าการแสดงออกของ P-gp expression ในเซลล์บุผนังหลอดเลือดฝอยในสมองลดลงในสมองส่วน striatum ที่แยกมาจากผู้ที่เป็นมาลาเรียขึ้นสมองทั้ง 3 ราย โดยเทียบกับกลุ่มควบคุม ในขณะที่เซลล์บุผนังหลอดเลือดฝอยในสมองมีการแสดงออกของ P-gp ลดลงใน cerebellum เพียง 2 รายจาก 3 ราย และใน brain stem hippocampus และ cerebral cortex พบการลดลงของ P-gp เพียง 1 ราย สำหรับกลุ่มที่เป็น encephalopathy โดยไม่มีการติดเชื้อ *P. falciparum* พบว่าการแสดงออกของ P-gp ลดลงในเซลล์บุผนังหลอดเลือดฝอยในสมองที่แยกจาก cerebellum, cerebral cortex, brain stem และ striatum แต่ไม่ลดลงใน hippocampus ผลการศึกษานี้แสดงให้เห็นถึงการแสดงออกของ P-gp ที่เปลี่ยนไปในมาลาเรียขึ้นสมอง ซึ่งมีรูปแบบที่แตกต่างกันในแต่ละส่วนของสมอง