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PALM OIL SUPPLEMENTATION PREVENTS FURTHER TYROSINE DEPHOSPHORYLATION OF ERYTHROCYTES 30-KDA PROTEIN DURING PLASMODIUM BERGHEI INFECTION

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ABSTRACT

This study was carried out to determine effect of palm oil (PO) supplementation on erythrocytes phosphotyrosine proteins in Plasmodium berghei (PB) infection. A number of 40 experimental mice were divided into observation (n=20) and sampling (n=20) groups which were then subdivided into four groups (group A: +PO+PB; group B: +PO-PB; group C: -PO+PB and group D: -PO-PB) with n=5 each. It was observed that parasitemia development of observation group was delayed in group A as compared to group C starting from day 7 post-infection onwards. Group A and C reached 40% parasitemia at day 13 and day 11 respectively. Immunoblotting of electrophoretically separated proteins revealed that there were four erythrocytes phosphotyrosine proteins present in all sampling groups namely 120-kDa, 60-kDa, 30-kDa, 17-kDa and 14-kDa. It was noted that PO supplementation did not affect all phosphotyrosine proteins in normal experimental mice apparently whilst PB infection affected 30-kDa phosphotyrosine protein significantly ($p<0.05$). Furthermore, PO supplementation was found to prevent further tyrosine dephosphorylation of 30-kDa protein by PB infection. We suggest that the delay of parasitemia development in infected experimental mice is due to prevention of tyosine dephosphorylation of 30-kDa protein.

KEY WORDS

Palm oil; malaria; Plasmodium berghei and tyrosine phosphorylation.

INTRODUCTION

Plasmodium is known to modulate protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) as their strategy for invasion and multiplying in erythrocytes of host [1] which results in existence of many phosphoproteins during malarial development. This has been supported by [2] reporting that tyrosine phosphorylation - dephosphorylation mechanism is crucial in malarial infection. The abnormal protein phosphorylation in malaria may cause destruction of erythrocytes which lead to hemolysis and anemia [3]. Thus, the understanding on involvement of tyrosine phosphorylation in malaria infection is crucial to improve the existing antimalaria treatment plan.

Plasmodium berghei is one of the malaria parasite infecting rodents. Others include *P. yoelli, chabaudi, P. knowlesi* and *P. vivax*. This unicellular protozoan which serves as a good model for the human parasites has a complex life cycle comprising of sexual and asexual stages [4]. A report by [5] stated that *P. berghei* strain ANKA and strain NK65 were isolated from *Anopheles dureni millecampsi* but in different forest galleries, River Kasapa and River Kisanga respectively. Those forest galleries are located in Lubumbashi, Republic of Congo. Other isolated

P. berghei strains are LUKA, K173, KSP11 and SP11. For many years, *P. berghei* has been a good model to investigate cellular signaling in malaria infection.

Palm oil (PO) is potent anti-oxidant rich oil which contains carotenoids, tocopherols, tocotrienols and lycopenes [4]. It is also high in palmitic (44 %) and oleic acid (40 %) [5]. The red palm oil is obtained from the fruit of the oil palm tree and is the most widely produced edible vegetable oil in the world. Its nutritional and health attributes have been well documented [6] while involvement of cellular signaling mechanism in PO-induced protection also has been demonstrated [7]. Therefore it is likely that various protective effects of PO are associated with complex molecular mechanism which remains to be elucidated.

Because tyrosine kinase signaling is important in *Plasmodium* infection [8], this study was carried out to determine effect of PO supplementation on the profile of erythrocytes phosphotyrosine proteins during *Plasmodium berghei* infection. In this study, there were two scopes investigated with regard to the role of PO in malarial infection namely: i) monitoring of intraerythrocytic parasitemia development in experimental mice for 20 days and ii) protein analysis on both normal and infected experimental mice when the latter reach their 40% parasitemia. The intraerythrocytic parasitemia in the experimental mice was induced by intraperitoneal injection while its development was monitored through microscopic analysis of Giemsa-stained blood smears. The protein analysis was to determine level of phosphotyrosine protein in erythrocytes of the experimental mice, which involved a combination of SDS polyacrylamide gel electrophoresis, immunoblotting and densitometry. Our study provided an insight into how the intraerythrocytic parasitemia development could possibly be controlled by PO supplementation.

MATERIALS AND METHODS

Experimental design and malaria induction

A total of 40 male HSD mice aged eight weeks were maintained in the animal house of Universiti Kebangsaan Malaysia, Bangi, Selangor. The experimental mice were divided into observation (n=20) and sampling (n=20) groups which were then subdivided into four groups (group A: +PO+PB; group B: +PO-PB; group C: -PO+PB and group D: -PO-PB) with n=5 each. The mice from group A and B were supplemented with PO (0.3ml/mice/day) orally for four weeks prior to inoculation of PB parasites whereby a volume of 100 µl of infected blood (1.25 x 10^6 parasitized erythrocytes, 125 dilution with Alsever solution) was injected into the experimental mice intraperitoneally. To monitor parasitemia development in group A and C, blood films were prepared from mice tail, stained with Giemsa and examined using light microscopy within 20 days.

Blood sampling for protein analysis

Blood sampling was conducted on the experimental mice of sampling group. At 40% parasitemia, both normal and infected experimental were sacrificed in an air-tight container under chloroform anesthesia. The blood sample was then withdrawn from abdominal aorta of experimental mice using one ml syringe and was kept in Vacutainer tubes containing EDTA as anticoagulant. To obtain erythrocyte fraction for protein analysis, the blood samples were centrifuged at 4,000 rpm for 10 minutes.

Protein fraction and protein analysis

Erythrocytes protein fraction was prepared as reported by [9] whereby acetone solvent was used to precipitate cytosolic proteins following lysis of the blood sample (erythrocyte fraction)

and precipitation of haemoglobin using HEPES lysis buffer (pH 8.0, contained 1 mM EDTA, 1Mm EGTA, 5% PMSF and 1 mM Na_3VO_4) and a mixture of etanol:butanol (60:40) respectively. Protein concentration was then determined using Bradford assay at a wavelength of 595 nm [10] whereby bovine serum albumin was used as a standard. An amount of 30 µg of erythrocyte protein was resolved by electrophoresis in a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis [11]. Subsequently, the resolved proteins and molecular mass marker were transferred from SDS polyacrylamide gel onto a nitrocellulose membrane [12] which was then followed by incubation in 5% gelatin (two hours, room temperature), overnight incubation with primer antibody (polyclonal anti-phosphotyrosine, 1000 dilution with 0.1% PBST, 4^0C) and two hours incubation with secondary antibody (HRP conjugated anti-IgG rabbit, 3000 dilution with 0.1% PBST, room temperature). Antibody binding was assessed by the enhanced chemiluminescence (ECL) method comprising of Reagent 1 (Enhanced luminal reagent) and Reagent 2 (Oxidizing reagent). Level of phosphotyrosine proteins were analyzed densitometrically at a wavelength range from 450 nm to 700 nm. The optical density (OD) values were expressed as the mean ± standard error measurement and T-test was performed to determine degree of difference between the groups where $p<0.05$ was considered significant. The protocol of protein analysis was performed in triplicates.

RESULTS

Intraerythrocytic parasitemia

It is established that when the malaria parasite infect the erythrocytes, they induce intraerythrocytic parasitemia development. The successful invasion of the malaria parasites is well associated with changes in erythrocyte morphology. **Figure 1** depicts intraeryhrocytic parasitemia development in infected experimental mice while **Figure 2** shows parasite morphologies detected inside the infected erythrocytes.

The microscopic observation on Giemsa-stained blood films demonstrated that the intraerythrocytic parasitemia level in group A and C was low **(<4%)** for the first six days post-infection while the highest parasitemia level (90±0.98%) was achieved by group A at day 20. It was also found that the parasitemia development of group A (both observation and sampling) was delayed as compared to group C starting from day 7 post-infection onwards. On the other hand, merozoite morphology was noted to be dominant in the first seven days post-infection. Trophozoite morphology started to become dominant at day 8 onwards whilst schizont morphology was found to be dominant when the parasitemia level reached approximately 50%. Group A and C reached 40% parasitemia at day 13 and day 11 respectively. It was a strong likelihood that the delay of parasitemia development was due to the antioxidant contents in PO which scavenge the free radical abnormally generated during PB infection.

Phosphotyrosine proteins

Tyrosine phosphorylation is a post-translational modification process occurs on protein molecules by adding phosphate (PO43-) group to tyrosine amino residues. This process is catalyzed by protein tyrosine kinase (PTK) while the removal of that phosphate group is dependent on dephosphorylation by protein tyrosine phosphatase (PTP). A common method to study tyrosine phosphorylation - dephosphorylation is a combination of SDS polyacrylamide gel electrophoresis and immunoblotting.**Figure 3** shows phosphotyrosine proteins detected in erythrocytes of both normal

and infected experimental mice. It was revealed that there were four erythrocytes phosphotyrosine proteins present in all sampling groups namely 120-kDa, 60-kDa, 30-kDa, 17-kDa and 14-kDa. It was also noted that PO supplementation did not affect all phosphotyrosine proteins in normal experimental mice apparently whilst PB infection affected 30-kDa phosphotyrosine protein significantly ($p<0.05$). Moreover, PO supplementation was found to prevent further tyrosine dephosphorylation of 30-kDa protein by PB infection. Densitometric analysis demonstrated that the OD values of 30-kDa phosphotyrosine protein were as follows: 0.95±0.08 (group A), 1.07±0.15 (group B), 0.78±0.9 (group C) and 1.11±0.09 (group D). The level of 30-kDa phosphotyrosine proteins of group C was significantly lower ($P<0.05$) than that of group D. It seems that the PO was to retain the tyrosine phosphorylated state of 30-kDa protein during PB infection.

DISCUSSIONS

PO supplementation as an important strategy to reduce severity of malaria

For many years, PO has been consumed in several countries in order to control severity of malaria. Due to the fact that PO is rich in antioxidants and able to reduce oxidative stress [4], the PO supplementation has been an important agenda to improve protection against malaria in pre-school children [13]. In that study, the effect of PO supplementation on malaria was investigated in 207 children (aged 0-60 months) who presented with fever in August-October 1999 at several hospital clinics around Ile-Ife, Western Nigeria. The parameters measured were anthropometric data, body temperature, parasitaemia and plasma C-reactive protein (CRP), retinol, carotenoids and tocopherols. It was observed that median plasma concentrations of both alpha-carotene (0.518 mumol/L) and beta-carotene (0.698 mumol/L) in the children were high. There were also some evidences that the PO supplementation associated with a lower severity of malaria. Therefore, it is in agreement with the *in vivo* data herein.

Protective effect of PO and cellular signaling mechanism

A number of studies have shown that PO exhibits effect on cellular signaling mechanism. According to [14] the PO supplementation improved cardiac function of animal and was mediated by cyclic nucleotide signaling. In particular, they demonstrated that the functional recovery in animal hearts was associated with an elevation in the cGMP level and decrease of cAMP level. Their finding suggested that dietary PO supplementation improved reperfusion function through mechanisms with respect to activation of the NO-cGMP and inhibition of the cAMP pathway. Furthermore, a study by [15] showed that the PO supplementation also modulated the phosphorylation mechanism. By using a combination of SDS polyacrylamide gel electrophoresis and immunoblotting, it was noted that p38 and PKB/Akt phosphorylation increased significantly during reperfusion when compared with control hearts. Besides, a significant decrease in JNK phosphorylation and attenuation of poly (ADP-ribose) polymerase cleavage were also observed in the PO-supplemented group during reperfusion. It was suggested that the dietary PO supplementation caused differential phosphorylation of the MAPKs and PKB/Akt during ischemia/reperfusion-induced injury. The role of antioxidant in cellular signaling cascade was also evidenced by [16] in which it is useful to explain molecular mechanism of disease development and prevention. In the context of our study, prevention of further tyrosine

dephosphorylation of 30-kDa protein of by PO may attribute to its role in controlling intraerythrocytic parasitemia development.

Antioxidant and its influence on tyrosine phosphorylation

The net cellular level of tyrosine phosphorylation is sustained by enzymes which catalyze the incorporation (protein tyrosine kinases) or removal (protein tyrosine phosphatases) of phosphate from tyrosine amino residues. With respect to modulation of tyrosine phosphorylation by phytochemical consitutents, there is a study that revealed the inhibition of tyrosine phosphorylation by an organic compound, kaempferol. According to [19], the kaempferol which is a flavonoid widely found in various natural sources such as onions and citrus fruits, exhibited cardioprotective effect via its antioxidant activity. In the primary cultured rat aortic vascular smooth muscle cells (VSMCs), it inhibited both c-fos mRNA expression and PDGF beta-receptor tyrosine phosphorylation which in turn inhibited phosphorylation of the downstream signal transduction of PDGF such as ERK1/2, Akt and PLC-gamma 1. In addition, another study reported the role of antioxidant in inducing tyrosine phosphorylation. In 2010, Kaspar and Jaiswal [20] investigated effect of antioxidant treatment on the nuclear translocation of Bach 1, a transcription regulator protein which is dependent on phosphorylation of tyrosine 486. It was discovered that Bach1 levels inside the nucleus returned to normal at 4 h after antioxidant treatment in the absence but not in the presence of protein synthesis inhibitor cycloheximide. They concluded that antioxidant-induced tyrosine 486 phosphorylation leads to nuclear translocation of Bach1. In conjunction with these, it was possible that the antioxidant contents of PO prevent further tyrosine dephosphorylation of erythrocytes 30-kDa proteins by activating PTK and inhibiting PTP during PB infection.

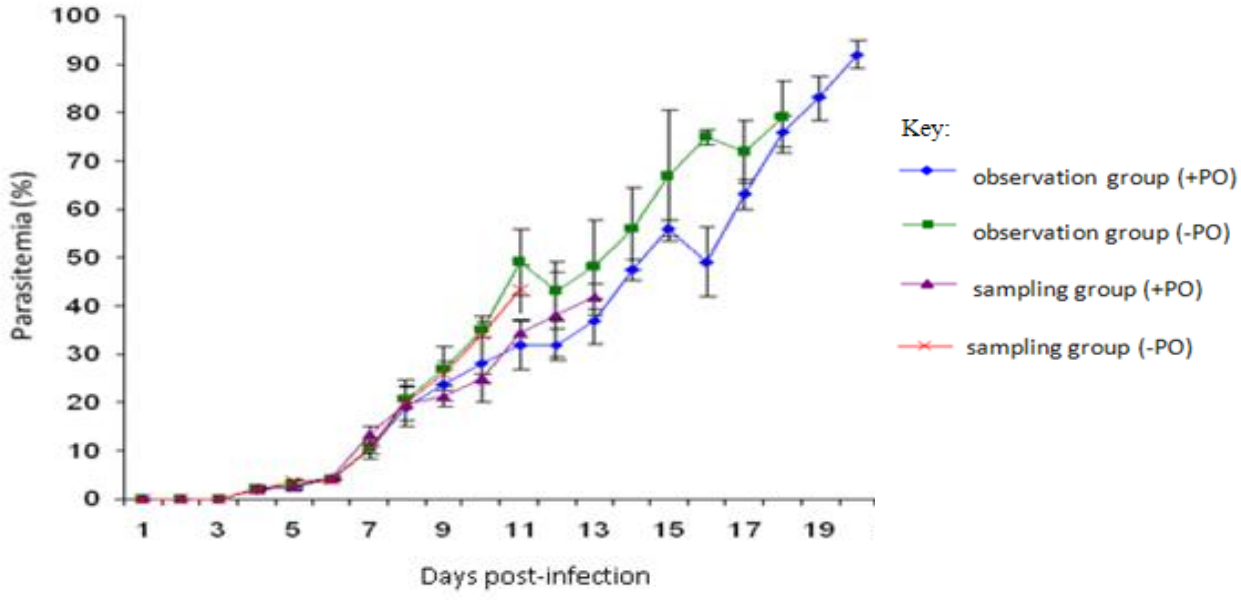


Figure1: Intraerythrocytic parasitemia development in infected experimental mice. Data are represented by mean ± standard error measurement (n=5).

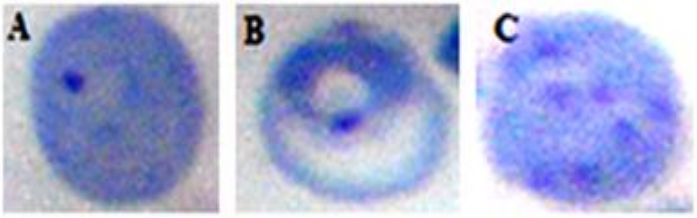
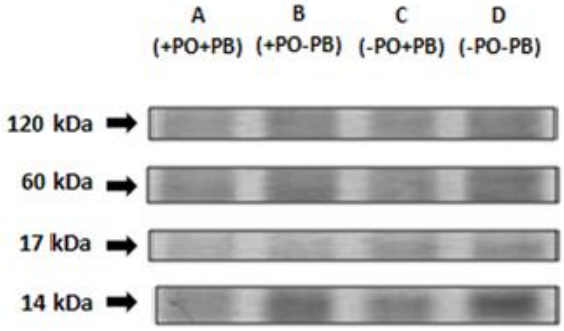
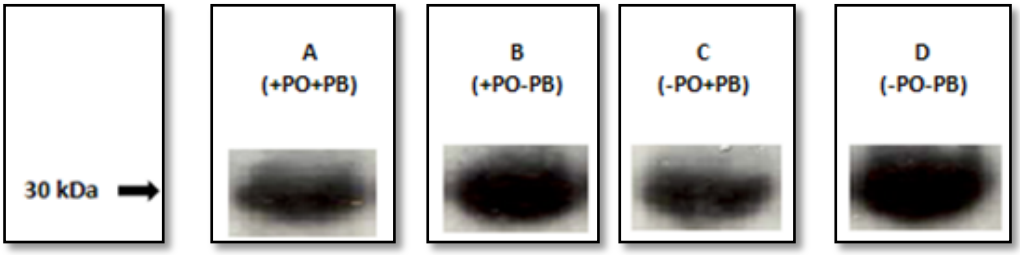


Figure 2: Parasite morphologies detected inside infected erythrocytes. A, B and C indicates merozoite, trophozoite and schizont respectively.





	Group A	Group B	Group C	Group D
OD value	0.95±0.08	1.07±0.15	0.78±0.31	1.11±0.09

Figure 3: Erythrocyte phosphotyrosine proteins in both normal and infected experimental mice. The phosphotyrosine protein bands were detected using primer antibody (polyclonal anti-phosphotyrosine) and secondary antibody (HRP conjugated anti-IgG rabbit). The intensity of phosphotyrosine protein bands were analyzed using densitometry method. Optical density values are represented by mean ± standard error measurement (n=5).

CONCLUSION

We have demonstrated that PO supplementation could delay the Intraerythrocytic parasitemia development which most probably due to its high antioxidant contents. Tyrosine dephosphorylation of 30-kDa proteins occurred following PB infection and it was substantially prevented by PO supplementation. It was proposed that the delay of intraerythrocytic parasitemia development in the infected experimental mice is due to prevention of further tyosine dephosphorylation of **30-kDa** protein.

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