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HEPATOPROTECTIVE BENEFIT OF PRANDIAL NATURAL COCOA POWDER ON VOLUME DENSITIES OF HEPATOCYTE ROUGH ENDOPLASMIC RETICULUM (RER) IN MURINE MALARIA

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Abstract
The hepatoprotective benefit of prandial natural cocoa powder (NCP) has been demonstrated in murine malaria, but the mechanism by which such benefit is achieved awaits determination. Investigating changes at the subcellular level can offer clues to understanding how cocoa protects hepatocytes in malarious rats. This research assessed the injurious effect of Plasmodium berghei (NK 65) infection on hepatocyte endoplasmic reticulum (ER) in rats and amelioration by prandial NCP. Twenty-four Sprague-Dawley rats were randomly assigned to four experimental groups of six animals. Rats in groups 1 (G1), 2 (G2), and 3 (G3) were infected by intraperitoneal inoculation with 0.2 ml of blood containing $1x10^5$ P. berghei (NK 65) parasites per μl. Rats in G1 and G2 were given unrestricted 24-hour access to 2% (weight/volume) NCP via water bottles for 14 days. Rats in G2 and G3 were given 0.6 ml of 4 mg/kg dispersible artemether-lumefantrine (AL) once every morning via oral gavage on days 4 and 5 post-inoculation, whilst G1 rats received 0.6 ml of aqueous NCP also via oral gavage (to equalize stress across the groups). Rats in Group 4 (G4) were uninfected controls and were given neither NCP nor AL. All rats were fed the same standard chow. The rats were euthanized, perfusion-fixed, and their livers dissected and processed for transmission electron microscopic (TEM) examination, fourteen days post-inoculation. G3 was found to have the highest increase (397%) in volume density of rough endoplasmic reticulum (RER) per area of hepatocyte (μm^2); followed by G2 (376%) and G1 (16%), relative to the uninfected control (G4). Serum levels of alanine transaminase (ALT) mirrored these RER differences in rat groups. Increased volume density of RER in hepatocytes indicative of intracellular stress was decreased 25-fold by ingestion of NCP compared to AL treatment, and 24-fold compared to co-treatment with AL and NCP ingestion.

Keywords
hepatoprotection, rats, *P. berghei*, antimalarial, nutraceutical, intracellular stress

Introduction

The WHO through its fact sheet on the world malaria report estimated the cases of malaria worldwide to be 198 million with an estimated 584,000 mortality of which 90% occurred in Africa in 2013 (WHO, 2018). The route of transmission of malaria in humans is similar to that in murine rodents. In brief, malaria infection in murine rodents is caused when *Plasmodium berghei* sporozoites, carried by the female anopheles mosquito *Anophelis stephensis*, are introduced into the rodent's body via a bite. At the bite site, the sporozoites enter a capillary and reach the liver either by the portal vein or hepatic artery Frevert et al. (2005). The sporozoites migrate through several hepatocytes causing cellular damage to each of the hepatocytes till they settle down in one hepatocyte, where they multiply and differentiate into several merozoites, which subsequently attack and damage red blood cells Frevert et al. (2005); Vaughan and Kappe (2017). *Plasmodium* is therefore an obligate intracellular parasite, which depends on host cell resources to enhance its survival and development (Inácio et al., 2015).

During intrahepatocyte replication and growth, the plasmodium parasite demands phospholipids to build its plasma membrane, endoplasmic reticulum (ER), and an extra-parasitic tubulovesicular network (TVN) necessary for transformation into merozoites (Burda et al., 2017; Grützke et al., 2014). The formation of merozoites increases the demand for phospholipids to form the required plasma membrane and therefore the parasite resorts to the host cell ER as well as the ER of the parasite itself as potential sources (Grützke et al., 2014).

Another way the liver stages of the malaria parasite co-opts hepatocyte responses to its benefit is by triggering the unfolded protein response (UPR) pathway (Inácio et al., 2015) which pathway is indispensable for parasite survival. It is notable that the UPR is turned on in all eukaryotes under circumstances that require expansion of the capacity of protein secretion which further triggers endoplasmic reticulum (ER) stress (Inácio et al., 2015). ER stress is known to be a protective mechanism that is meant to restore homeostasis by increasing the protein folding capacity within the ER and therefore temporarily decreasing transcription and translational response. A prolonged ER stress, however, could lead to an inflammatory response and apoptosis (Malhi and Kaufman, 2011). The *Plasmodium sp.* has adaptive mechanisms to counteract the negative effects of ER stress in infected hepatocytes by protecting the host cells from apoptosis via increasing levels of regulator proteins in the Bcl-2 family (Kaushansky et al., 2013a,b) and by inhibiting the activities of pro-inflammatory markers such as nuclear factor kappa β (NFkβ) directly by the major parasite surface protein, the

circumsporozoite protein (Singh et al., 2007).
Hepatocytes perform several metabolic processes, including protein synthesis and metabolism of lipids, carbohydrates, and micronutrients such as iron (Protzer et al., 2012). Many of these processes occur in the lumen or membrane domains of ER. As such, hepatocytes are usually rich in both smooth and rough ER. The UPR of the ER is an elaborate stress signaling pathway that is activated when ER function is challenged vis-à-vis increased demand on its synthetic capacity beyond what it can accommodate, leading to ER stress (Ron and Walter, 2007). A school of thought holds that ER stress contributes to parasite growth by controlling the metabolism of hepatic phosphatidylcholine which is subsequently taken up by the liver stages of plasmodium (Itoe et al., 2014). Host pathways disrupted by the parasite therefore, represent potential points of susceptibility and likewise desirable targets for the development of host-based malaria interventions (Inácio et al., 2015).
One of the major setbacks in malaria control and elimination efforts is drug resistance by the parasites (Petersen et al., 2011). For this reason, the World Health Organization (WHO) recommends the use of artemisinin in combination with a partner drug for the therapeutic treatment of uncomplicated malaria. The rationale is that fast-acting artemisinin protects the partner drug against parasite resistance (Daniyan et al., 2019). However, the emergence of resistance against artemisinin continues to be reported especially in some parts of Southeast Asia (Woodrow and White, 2017). Thus, artemisinin faces reduced efficacy and hence, the need for identifying novel antimalarials. It is known that antimalarials cause oxidative stress, and it, in turn, induces parasite heat shock mechanisms that augment resistance to antimalarial drugs (Akide-Ndunge et al., 2009). Sadly, it has been shown that none of the clinically approved anti-malarial drugs tested are inhibitors of the translation of heat shock proteins in plasmodium, although translation offers a potentially useful therapeutic target (Sheridan et al., 2018). Nutraceuticals such as cocoa as a form of prophylaxis hold promise for a better antimalarial action via multiple mechanisms that make the development of resistance less likely. Indeed, prandial natural cocoa was espoused as a diet-mediated antimalarial prophylaxis based on anecdotal reports and the multi-mechanistic potential of its manifold dietary nutrients (Addai, 2010). Limited studies have since validated some of the proposed mechanisms of cocoa's antimalarial activity. These include parasite inhibition (Amponsah et al., 2012), immune-modulation (Aladesemipe et al., 2013); attenuation of plasmodium-induced hepatocyte damage (Aidoo et al., 2012); attenuation of hepato-toxicity by high dose AL (Asiedu-Gyekye et al., 2016); and inhibition of parasite invasion of RBCs and sorbitol-induced haemolysis of infected erythrocytes (Adika et al., 2016). To promote wider adoption of NCP as one of the antidotes to malaria, it is needful to better understand the mechanism by which cocoa antimalarial effects are exerted. This study sought to contribute to this effort.

Materials and Methods

Animals and parasites

Twenty-four male Sprague Dawley rats aged 6 – 8 weeks and weighing 180 g – 200 g were kept at the University of Ghana Medical School Animal House, Korle Bu, Accra, Ghana. All the animals were kept under the same laboratory conditions of temperature (28°C ± 2°C), relative humidity (70% ± 4%), 12-hour light and dark cycle, and 24-hour natural ventilation. The rats were randomly assigned to 4 experimental groups of 6 animals each and kept in four different cages with dimensions of 30 cm x 22 cm x 16 cm (length x breadth x height respectively). The rats underwent 7 days of acclimatization in the infectious experimentation unit after being transferred from the animal holding unit. Before and during the study, their body weights were recorded and were fed the same rat chow from Ghana Agro Food Company (GAFCO, Tema, Ghana), and given autoclaved tap water every morning. Procedures involving the care and use of animals conformed to local institutional guidelines and complied with national and international guidelines for the use of animals in biomedical research. *P. berghei* (NK65) was donated by the Immunology Department of the Noguchi Memorial Institute for Medical Research (NMIMR), Ghana. The parasites in (infected) rat erythrocytes at a concentration of 1 x 106 were suspended in sodium citrate and stored in liquid nitrogen.

Experimental protocol

Rats in group 1 (G1) were inoculated with 0.2 ml of parasitized blood containing $1x10^5$ *P. berghei* (NK 65) parasites per µl of blood and given free access to aqueous NCP in water bottles *ad libitum* for 14 days. These rats were also given 0.5 ml to 0.8 ml of 2% (weight/volume) of the aqueous NCP by oral gavage once every morning per their body weights in order to equalize any stress that might have been experienced by the rats which were given AL by oral gavage on days 4 and 5 post-inoculation.
Rats in group 2 (G2) were similarly inoculated with 0.2 ml of parasitized blood containing $1x10^5$ *P. berghei* (NK 65) parasites per µl of blood and given free access to aqueous NCP *ad libitum* for 14 days as well as 0.6 ml of 20 mg/ 120 mg AL (Coartem, Novartis Pharma AG, Basel, Switzerland) by oral gavage once daily on days 4 and 5 post-inoculation. Rats in group 3 (G3) were inoculated with the same volume and concentration of *P. berghei* (NK 65) parasites as well as the same AL treatment on the same days as for G2 rats; however, this group was not given NCP but water *ad libitum* for 14 days. Rats in group 4 (G4), which served as the control group were neither inoculated with parasites nor given aqueous NCP or AL but were given water ad libitum and the same by gavage on days 4 and 5.

Preparation of the parasite inoculum

The protocol followed has been described previously (Aidoo et al., 2012). Briefly, cryopreserved parasites were taken through routine procedures to prepare an inoculum in a com-

plete parasite medium (CPM) (Gibco, USA). After inoculation of the stock (donor) with parasites, a series of passages were run in subsequent donor rats to establish infection. The establishment of infection was confirmed by examination of thick and thin films prepared from the tail veins of the rats from which parasite density and percentage of parasites were calculated. Parasite density per μl of blood was determined by counting parasites against the total WBCs (~200 WBCs) in Giemsa-stained thick blood films and the figure was multiplied by 8000 (the standard WBC count per μl of blood). It was calculated as follows:

$$Parasite\ density = \left[\left(\frac{number\ of\ parasites}{200WBCs}\right) \text{x } 8000\right] \quad (1)$$

Parasitaemia was monitored every two days post-inoculation by Giemsa-stained thin blood films from tail veins and expressed as a percentage of at least 500 RBCs. Between 500 – 1000 RBCs were counted per slide with a mechanical hand tally counter (H-104, USA) and percentage parasitaemia was calculated as follows:

$$Parasite\ \% = \left[\left(\frac{number\ of\ infected\ RBCs}{total\ number\ of\ RBCs\ counted}\right) \text{x}100\right] \quad (2)$$

At 45% parasitaemia, parasitized blood was drawn from donor rats by cardiac puncture with a 23 gauge hypodermic needle containing 0.2 mL of trisodium citrate (as an anticoagulant). The donor rats were anaesthetized with diethyl ether (AVONCHEM, Wellington House, Waterloo St. West Macclesfield, Cheshire, UK). The blood was then put into Eppendorf tubes (Reagiergefab, Sarstedt Aktiengesellschaft and Co., Germany) containing 1.5 ml of normal saline for dilution. The diluted blood was transferred into a 15 ml falcon tube (Rohrchen Greiner bio-one, Germany) containing 2.0 ml of trisodium citrate to prevent clotting while inoculation was done. The rats were individually inoculated intraperitoneally (i.p.) with 0.2ml of the diluted parasitized blood containing $1 \text{x} 10^5$ *Plasmodium berghei* (NK65) parasites per μl of blood.

Preparation of 2% (w/v) NCP

NCP was prepared [as previously described (Aidoo et al., 2012)] with 2.0 g of commercially obtained NCP (GoodFood®, Kakawa Ent. Ltd. Ghana, batch no. DA1402A) dissolved in 100 ml of freshly boiled tap water. The mixture was stirred with appropriate vigour until frothing indicative of uniform suspension was observed. It was then cooled under running tap water. Fresh NCP suspension was prepared daily throughout the experiment and G1 and G2 rats drank volitionally via water bottles 7 days before inoculation of parasitized RBC and throughout the 14 days post-inoculation. Administration of 0.5 ml – 0.8 ml of NCP by oral gavage was done to equalize the stress to rats given AL by gavage and was given on the same day as AL administration.

Artemether lumefantrine (AL) administration

A 0.6ml of 20 mg/120 mg dispersible AL (Coartem, Novartis Pharma AG, Basel, Switzerland) purchased from a licensed chemist was administered via oral gavage once each morning to the rats in G2 and G3 groups on the 4th and 5th days after parasite inoculation after thin and thick blood films confirmed the presence of parasites on day 3 post-inoculation.

Preparation of rats for liver harvesting

All animals were sacrificed on day 14 post-inoculation. Each animal was euthanized by diethyl ether (AVONCHEM, Wellington House, Waterloo St. West Macclesfield, Cheshire, UK) inhalation in an anaesthesia jar followed by perfusion fixation. Pain reflex tests (Rat Hands-on Laboratory, University of Washington) (Animal Use Training Sessions, 2021) were performed to assess the anaesthetic depth of each rat before the commencement of perfusion. Perfusion was performed intracardially via gravity by canulating the left ventricle with a hypodermic needle (23 gauge) attached to the blood-given set. The right atrium was punctured to allow effluent flow. Perfusion was started with normal saline until the liver turned pale, then followed with fresh fixative (solution of 2% formaldehyde and 2% glutaraldehyde buffered at pH 7.4 with 0.1M cacodylate). Adequacy of perfusion was determined when the liver was firm when touched with a pair of forceps.

Systematic uniform random sampling (SURS) of the liver for stereology

Following perfusion-fixation, the liver of each rat was excised in whole from the animal and carefully separated into the right, left, median, and caudate lobes. Using a disposable microtome blade, each lobe was sliced into 1.0 mm^3 thickness. Seven (7) liver slices were obtained from each of the right, left, and median lobes; whilst the caudate lobe yielded five (5) slices because it was the smaller of all the lobes. Representative samples were systematically selected for each rat liver by picking every 2nd, 4th, and 6th slice from the larger lobes, and the 2nd and 4th slice from the smaller caudate lobe (Fig 1).

Processing and random sampling for Transmission Electron Microscopy (TEM)

The systematically sampled slices of liver were taken through routine TEM tissue processing protocol of post-fixation in 3% cacodylate buffered glutaraldehyde for 3 hours, followed by washing in two 10-minute changes of 3% cacodylate buffer. Thereafter, each liver slice was further cut into about 10 – 15 mesh-like thin slices with a razor in a petri dish and further fixed in 2% glutaraldehyde for 1 hour. Three (3) out of each group of 10 – 15 mesh-like slices were randomly selected to represent each lobe. The slices were then dehydrated in graded alcohols, cleared with propylene oxide and subsequently embedded in epoxy resin (Chiyoda Junyaku Inc; Japan and LAAD Research Industries Inc; USA). Ultrathin sections (70 nm) were cut with a Leica Ultramicrotome (Leica Company, Austria), mounted on a copper grid, stained with uranyl acetate, and observed in a TEM (JEOL JEM – 1010, JEOL LTD, Japan).

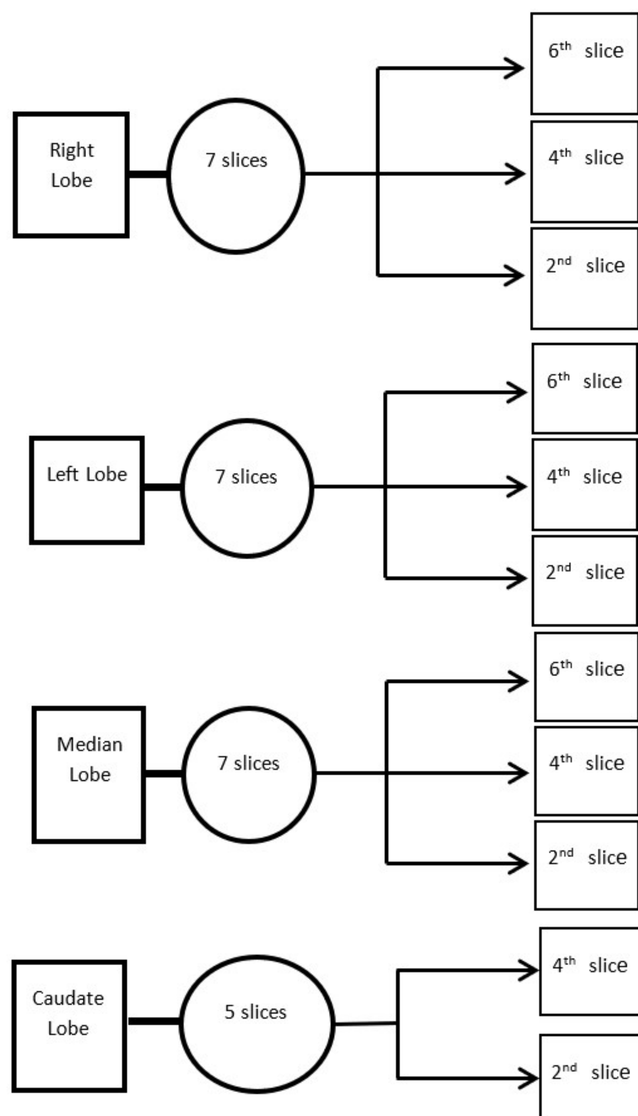

Figure 1. Chart illustrating the procedure for systematic uniform random sampling (SURS) of liver lobes used in this study.

Japan).
The developed films were later scanned by a special negative film scanner (Prime Film 7200, Pacific Image Electronics Co., LTD, Taiwan) together with another negative film scanner application (HELMUT film scanner, dk.codeunited.helmut.apk). A design-based stereological system i.e. the test-system for grid-point counting (Weibel 1979) was used to estimate the volume density (Vv) of RER per hepatocyte area (μm^2). A test area of 58 mm x 80 mm was computer-generated by Adobe Photoshop CS6 extended version and was superimposed on the micrographs with total points (Pt) of 4,640 (Fig. 2). The partial points (Pp) of RER per hepatocyte area (μm^2) was determined by clicking all RER hitting the test grid with the left mouse button and recorded as (Pp). The volume density of RER per hepatocyte area (μm^2) was calculated as a ratio of (Pp) and (Pt) (Weibel 1979). Several different fields of interest were systematically and randomly captured and the averages of these measurements were computed as described earlier.

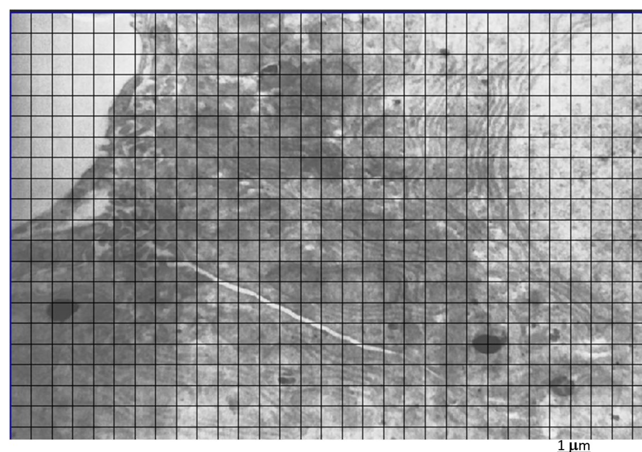

Figure 2. A transmission electron micrograph of rat liver superimposed with a lattice test grid for counting RER. Stain: uranyl acetate.

Stereological determination of cellular ultrastructural variables as indicators of plasmodial injury to the liver

The objective of this study was to quantitatively estimate ultrastructural indicators of liver injury (i.e. volume density of RER) coincident with malaria parasitaemia and the degree to which prandial natural cocoa ameliorated the damage or alteration. To accomplish this, a design-based stereological procedure was applied to transmission electron micrographs of the systematically sampled liver tissues.
The photo tool of the electron microscope (JEOL JEM-1010, JEOL LTD, Japan) (APPENDIX XII) was used to systematically randomize the generation of micrographs of rat livers onto a negative film which was developed using the COPINAL microfilm developer (Fuji Photo Film Company LTD, Tokyo, Japan).

Biochemical assays

Between 10 ml to 15 ml of blood was collected transcardially using a 20 ml hypodermic needle attached to a 21G needle (BDH, England) from each euthanized rat before perfusion. The blood was put in serum separators and then centrifuged at 10,000 rpm for 10 minutes. The activity of serum ALT and AST were assayed with an automated biochemical analyzer (Flexor Lab E, VITA Scientific, Netherlands) at NMIMR, Legon Ghana.

Statistical analysis

GraphPad Prism 7 was used for analyzing data obtained from the study. One-way analysis of variance (ANOVA) and Kruskal-Wallis tests were performed followed by Bonferroni and Dunn's posthoc multiple comparisons respectively to identify the group(s) eliciting difference(s). All data were

expressed as mean and standard deviation (SD); P values < 0.05 were considered significant.

Results and Discussion

Results

Out of the six rats in each group, two rats in Group 1 and a rat each in Groups 2, 3, and 4 died on different days in the course of the 14-day experiment. Hence the sample size for Groups 1, 2, 3, and 4 stood at 4, 5, 5, and 5 rats respectively.

Percentage of parasitized RBCs in infected rat groups

The percentage of parasitized RBCs in infected rat groups obtained as a result of counting infected RBCs from thin blood films prepared from tail veins of the infected rat groups is represented graphically (Fig. 3). One-way ANOVA and subsequently post hoc analysis showed significant differences ($p < 0.0001$) between the groups on same-day comparisons. However, there was no significant difference between the groups after the 7th day post-inoculation (Table. 1).

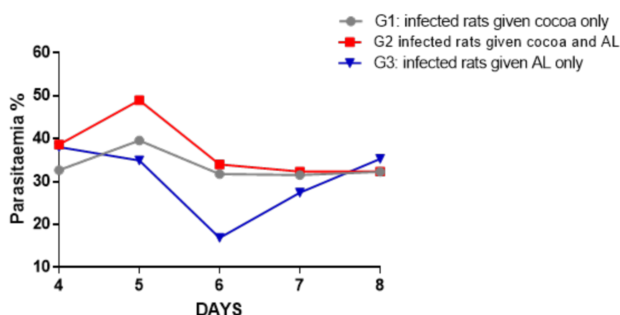

Figure 3. Line plot of the trend of parasitaemia of parasitized RBCs in the infected rat groups from days 4-8 post-inoculation.

Table 1. Bonferroni's multiple comparison tests of percentage parasitaemia among the rat groups

Groups	t	95.00% CI of diff.	p-value
G1 D4 vs. G2 D4	3.087	-33.99 to 2.962	0.4061
G1 D4 vs. G3 D4*	3.703	-29.81 to -0.1059	0.0452
G2 D4 vs. G3 D4	0.102	-19.51 to 20.62	>0.9999
G1 D5 vs. G2 D5***	6.417	-43.33 to -11.76	<0.0001
G1 D5 vs. G3 D5	3.425	-27.99 to 0.9934	0.1271
G2 D5 vs. G3 D5**	4.167	1.651 to 26.45	0.007
G1 D6 vs. G2 D6	0.678	-14.42 to 9.933	>0.9999
G1 D6 vs. G3 D6**	4.519	2.771 to 26.97	0.0015
G2 D6 vs. G3 D6***	5.740	6.151 to 28.08	<0.0001
G1 D7 vs. G2 D7	0.2482	-12.26 to 10.71	>0.9999
G1 D7 vs. G3 D7	1.232	-8.216 to 16.5	>0.9999
G2 D7 vs. G3 D7	1.722	-5.578 to 15.41	>0.9999
G1 D8 vs. G2 D8	2.149	-28.56 to 7.49	>0.9999
G1 D8 vs. G3 D8	2.321	-29.86 to 6.75	>0.9999
G2 D8 vs. G3 D8	0.3205	-12.7 to 10.66	>0.9999

*CI: confidence interval; *: significant difference*

Volume densities of RER per area of hepatocyte (μm^2)

The mean volume densities of RER per area of hepatocyte (μm^2) are shown in figure 4. One-way ANOVA showed a significant difference ($p < 0.05$) between the groups and this was confirmed by the post hoc Bonferroni analysis (Table 2).

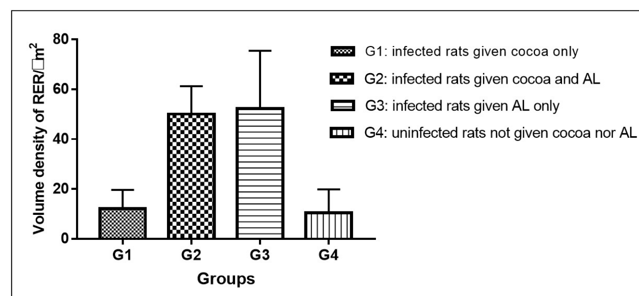

Figure 4. Bar chart showing the volume densities of RER per area of hepatocyte (μm^2) in the treatment groups. Error bars represent standard deviation. Level of significant difference by Bonferroni post hoc test: G1/G2: p = 0.003, G1/G3: p = 0.002, G2/G4: p = 0.004, G3/G4: p = 0.003

Table 2. Bonferroni multiple comparison test of concentration of RER per area of hepatocyte (μm^2) in the 4 rat treatment groups

Groups	t	p-value	95.00% CI of diff.
G1 vs G2**	4.41	p = 0.003	-63.62 to - 12.04
G1 vs G3**	4.67	p = 0.002	-65.82 to -14.24
G1 vs G4	0.18	p > 0.999	-25.82 to 29.16
G2 vs G3	0.25	p > 0.999	-29.14 to 24.74
G2 vs G4**	4.16	p = 0.004	10.93 to 68.07
G3 vs G4*	4.39	p = 0.003	13.13 to 70.27

*CI: confidence interval; *: significant difference*

Biochemical markers for liver function

One-way ANOVA performed on the levels of serum ALT (U/L) among the rat groups showed a significant value (p = 0.003) (Fig. 5). A Bonferroni multiple comparisons confirmed a significant difference between G2 : G4 (p = 0.019) and between G3 : G4 (p = 0.002) but no significant difference between G1 : G4. There was no significant difference in AST (U/L) among the rat groups (p = 0.88, F = 0.21, with 3 degrees of freedom) (Fig. 6).

Discussion

As a sequel to an earlier study by the author at the light microscopic level which demonstrated that liver damage was mitigated by prandial NCP in mice infected with *P. berghei*, the present study focused on ultra-structural volume densities of RER in plasmodium-infected rats. RER is increasingly receiving attention as an attractive potential therapeutic target because it is apparent that maintaining ER function and reducing ER stress may be able to prevent metabolic diseases (Inácio et al., 2015). Results from our present work offer a paradigmatic quest for making the liver stages a promising

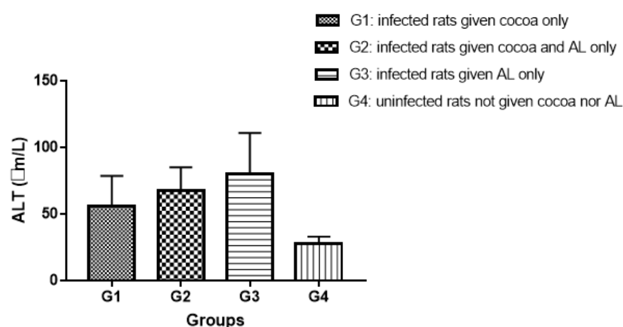

Figure 5. Bar chart of levels of serum ALT (u/L) activity in rats of the 4 treatment groups studied. G1: infected rats given cocoa only, G2: infected rats given cocoa and AL, G3: infected rats given AL-only, G4: uninfected rats not given cocoa nor AL. Error bars represent standard deviation (SD). Level of significant difference by Bonferroni post hoc test: G2/G4: p = 0.019, G3/G4: p = 0.002.

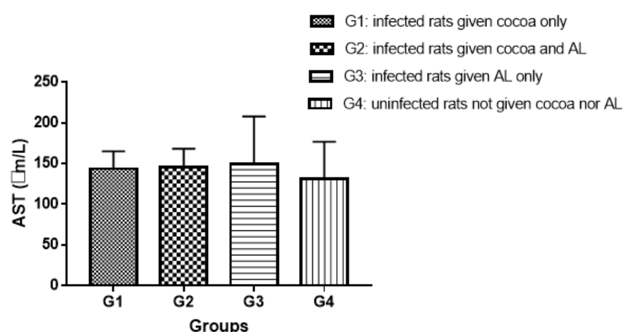

Figure 6. Bar chart showing levels of serum AST (u/L) activity in rats of the 4 treatment groups studied after plasmodial inoculation. G1: infected rats given cocoa only, G2: infected rats given cocoa and AL, G3: infected rats given AL-only, G4: uninfected rats not given cocoa nor AL. Error bars represent deviation (SD).

target for malaria control, and present NCP as an effective therapeutic agent via this mechanism.

Our results demonstrated an almost four hundred-fold (397%) increase of RER volume density in hepatocytes of plasmodium-infected rats given AL treatment compared with the density of the organelles in liver cells of uninfected (control) rats. Remarkably, in plasmodium-infected rats that voluntarily drank aqueous NCP without AL treatment, the hepatocyte RER volume density increased by a measly 16% in comparison with control rats. Equally, parasite-infected rats with similar free access to NCP but given AL co-treatment had their hepatocytes RER volume density being 21-fold less than in AL-only treated rats and 376% increase compared to control animals. It is instructive that parasite densities were not significantly different in the four rat groups after day 7 of the 14 days of experimentation.

The liver stage of plasmodium preferentially develops in the host juxta-nuclear region and its parasitophorous vacuole membrane (PVM) appears to form an association with the host ER that allows the passage of molecules from the hepatocyte to the parasite (Bano et al., 2007)(Bano et al. 2007).

There seems to be a beneficial role of the host ER stress pathway for *Plasmodium* survival in infected hepatocytes as explained earlier. It is plausible that as an adaptive mechanism to cope with increased synthesis to accommodate increasing parasite demand, the hepatocyte RER increases in volume. The availability of more phospholipids in the increased volume of RER will benefit the parasites' need for more plasma membranes for the thousands of merozoites maturing in the hepatocyte. Moreover, the RER volume density increase is apparently not mitigated, but perhaps exacerbated, by possible extra stress imposed by detoxification of AL despite its benefit in killing *plasmodium*.

Proteins and transcripts that play a critical role in the UPR are elevated in hepatocytes in response to infection (Inácio et al., 2015); possibly to regulate lipid metabolism as a way to support parasite growth. It has been postulated that ER-resident UPR is activated in host hepatocytes upon *P. berghei* infection, which also induces the expression of XBP1s (the active form of the UPR mediator XBP1) as well as CREBH, which activities *in vivo*, increase *Plasmodium* liver infection (Inácio et al., 2015). ER stress and UPR have also been reported to be activated in specific liver diseases such as steatosis and viral hepatitis (Malhi and Kaufman, 2011). A transcriptomic analysis of *P. berghei* sporozoite-infected mouse Hepa 1.6 cells at different time points (Albuquerque et al., 2009) showed transcriptional upregulation of several key ER stress markers as early as 6 hours after infection (Inácio et al., 2015).

Hepatoprotection was shown in previous work (Aidoo et al., 2012), and in the present study, prandial NCP was associated with a significant diminution of increasing volume density of RER compared with the AL-treated rats. It is known that oxidative damage and reduced glutathione levels trigger the activation of cell organelles such as mitochondria and RER which subsequently affect the normal functioning of these cell organelles (Lash, 2006). It has been cited by Guicciardi et al. (2013) that signaling between endoplasmic reticulum and mitochondria promotes hepatocyte apoptosis in response to excessive free fatty acid generation akin to what was observed in plasmodium-infected mice not given cocoa in a previous study (Aidoo et al., 2012). Based on these findings, there is a strong suggestion in our present results that prandial NCP minimized hepatocyte injury by impeding parasite-induced hyperactivity of RER including apoptotic pathway signaling. Moreover, the 25-fold greater volume density of RER in infected rats given only AL compared to the prandial NCP rat group may support the postulate that hyperactivation of cell organelles is a possible trigger of innate immune system activation (Pérez-Cano and Castell, 2016). Concerning our present report, we hypothesize that cocoa dietary nutrients resisted deleterious activation of the immune system in plasmodium-infected rats, by significantly minimizing RER hyperactivation. There is support for this speculation from several reports that dietary

flavonoids modulate inflammatory and oxidative responses, whereas malaria hepatic dysfunction is characterized by an increase in the serum levels of aminotransferases (Aidoo et al., 2012; Monagas et al., 2009; Vázquez-Agell et al., 2013). The present study also found that compared to the control rats, infected rat groups that were not given NCP had higher ALT (a proxy inflammatory marker) activity.
Taken together, our findings corroborate the thesis that potent anti-inflammatory and antioxidant properties of dietary nutrients in cocoa enable it to substantially minimize hepatocellular damage arising from plasmodial infection (Chiva-Blanch et al., 2012; Del Rio et al., 2013). Cocoa is one of the richest sources of antioxidants and is ranked one of the foods containing the highest antioxidant capacity (Khan et al., 2014). There is high content of flavonoids in cocoa besides other polyphenols such as quercetin, isoquercitrin, naringenin, luteolin, and apigenin (Amedonu et al., 2015). The bioavailability and bio-efficacy of polyphenols in animal models and humans have been extensively reviewed (Del Rio et al., 2013; Khan et al., 2014). Cocoa is rich in quercetin and quercetin is easily absorbed by the rat stomach (Crespy et al., 2002). Absorbed flavanols are highly distributed and can be found in the liver, spleen, thymus, testes, and mesenteric lymphoid nodules (Urpi-Sarda et al., 2010). Malaria hepatocellular dysfunction is characterized by an increase in the serum concentrations of bilirubin, aspartate, and alanine transaminases (Aidoo et al., 2012; Monagas et al., 2009; Vázquez-Agell et al., 2013) suggestive of inflammatory stress. However, flavonoids, found in cocoa, modulate inflammation and certain immunological pathways (Aidoo et al., 2012; Chiva-Blanch et al., 2012; Cooper et al., 2008). It is proffered from our results of relative volume densities of RER (Fig. 4 and table 2) that, resistance of RER hyperactivation may be a possible mechanism by which prandial NCP ameliorates hepatic damage by plasmodium and/or artemether-lumefantrine (Asiedu-Gyekye et al., 2016). Hence, it is affirmed that cocoa is a promising adjuvant to the antiplasmodial drugs to ameliorate hepatotoxicity.

Conclusion

Increased volume density of RER in Plasmodium infection is ultrastructural evidence of adaptive response to increased RER stress. Consumption of aqueous NCP mitigated this stress response in RER during plasmodial infection. It is postulated that anti-inflammatory and antioxidant properties of NCP dietary nutrients are responsible for the reported RER stress mitigation This finding is important in efforts to elucidate the antimalarial activity of NCP, besides providing impetus for future research to better understand the implications of the RER-Plasmodium interaction as a target in therapy.

Ethical Approval

This study was independently approved by the Committee on Research, Publications and Ethics of the Kwame Nkrumah University of Science and Technology, School of Medical Sciences; and the Institutional Animal Care and Use Committee (IACUC) of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra.

- **Data availability:** No data availability applies.

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- **Number of tables and figures:** There are 2 tables and 6 figures.

- **Conflict of Interest:** Frederick K. Addai is a non-salaried Director of Kakawa Enterprise Limited. No other author has any conflict of interest to declare.

Author contributions

- **Eric Aidoo** conceived the idea and design of the work, drafted the manuscript, acquired, analyzed and interpreted the data, drafted/reviewed the manuscript and approved it for publication; obtained funding from the University of Cape Coast, Training & Development Unit. This work formed part of his Ph.D. research.

- **Prof. Frederick Kwaku Addai** substantially contributed to the conception and design of this work; critically reviewed the draft of this manuscript and approved the final version for publication. He was the lead supervisor of the Ph.D. project.

- **Prof. Phyllis Addo** contributed to the design of this work; advised on the animal experimentation, and statistical analysis, and approved the final version for publication.

Abbreviation

AL	Artemether Lumefantrine
ALT	Alanine Transaminase
CREBH	Cyclic AMP-Responsive Element-Binding protein H
ER	Endoplasmic Reticulum
IACUC	Institutional Animal Care and Use Committee
NCP	Natural Cocoa Powder
NFkβ	Nuclear Factor kappa β
PVM	Parasitophorous Vacuole Membrane
RER	Rough Endoplasmic Reticulum
TEM	Transmission Electron Microscope
TVN	Tubovesicular Network
UPR	Unfolded Protein Response
WHO	World Health Organization
XBP1	X-Box Binding Protein 1

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