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Ultrastructural hepatic damage in murine malaria with and without prandial natural cocoa powder and artemether-lumefantrine treatment

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Abstract

Background: There is paucity of data on the potential benefit of cocoa in reducing oxidative stress and inflammation in the pathophysiology of plasmodial infection and its associated damage to liver tissues

Objective: This study compared hepatocyte ultrastructural integrity in rodent malaria treated with aqueous natural cocoa powder (NCP) ingestion with and without artemether-lumefantrine (AL).

Methods: Twenty-four Sprague-Dawley rats were randomly assigned to groups. Every rat was inoculated with 0.2 ml of parasitised blood containing 1 x 10^5 *P. berghei* (NK 65) parasites per microlitre to induce malaria infection. Subsequently, group 1 rats were given 2% (weight/volume) aqueous NCP; group 2 rats were given 2% NCP and 0.6 ml of 4.0 mg/kg AL. Group 3 rats received only the same dose of AL as group 2, whilst group 4 rats were given neither NCP nor AL. NCP and AL were administered by oral gavage once daily. Liver tissue harvested from euthanised and perfusion-fixed rats was processed for transmission electron microscopic examination. Hepatic tissue damage was quantitatively assessed using design-based stereology. Ultrastructural variables assessed were sinusoidal diameters, sinusoidal endothelial wall thickness, volume density of Kupffer cells, and perisinusoidal microvilli.

Results: As per the study variables, liver damage in group 1 rats was significantly extenuated compared with rats in group 2, group 3, and group 4. Serum biochemical markers assayed indicated statistically lower levels of aspartate transaminase (AST) and alanine transaminase (ALT) in groups 1,2 and 3 rats compared to group 4 rats. Inferably, mitigation of liver ultrastructural damage in *P. berghei*-infected rats given NCP was better than treatment with AL and putatively attributable to the anti-inflammatory activity of cocoa evidenced by significantly lower serum transaminases.

Conclusion: The evidence shows that damage to the ultrastructural liver morphology in murine malaria was significantly mitigated by daily ingestion of NCP compared with AL treatment with respect to hepatic sinusoidal endothelial thickness and density of hepatic microvilli despite parasitaemia being comparable to untreated control rats

Keywords: Hepatoprotection, cocoa, murine, plasmodial infection, stereology

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INTRODUCTION

The 2019 factsheet of the US Center for Disease Control and Prevention (CDC) on Malaria's Impact Worldwide [1] cites the World Health Organization's World (WHO) Malaria Report 2017 that malaria is one of the most severe public health problems worldwide. Furthermore, nearly half the world's population lives in areas at risk of malaria transmission, and it is the leading cause of death and disease in many developing countries. Drug resistance of plasmodium presents an enduring challenge for the global curtailment of the malaria scourge [2]. This necessitates a continued search for novel treatment options, of which nutraceuticals such as cocoa hold promise [3]. Despite strong reservations about the realistic transfer of laboratory studies on rodent malaria to clinical trials, there remains wide recognition of the important contribution of animal models in malaria research [4-6]. Extensive literature on the pathophysiology

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of malaria [7-12] indicates that the plasmodium parasites first damage infected erythrocytes directly and then trigger a chain reaction of nonspecific inflammatory processes, which elicit host immunological responses that further aggravate the inflammatory reactions. In rodent malaria infection, *plasmodium berghei* sporozoites reach the liver either by the portal vein or hepatic artery and migrate through several hepatocytes, causing cellular damage [13]. The parasites eventually invade one hepatocyte, where they multiply and differentiate into thousands of merozoites, which subsequently attack and damage red blood cells. *Plasmodium berghei* infection results in chronic oxidative stress, and tissue damage is a known cause of reactive oxygen species [14-16]. Cocoa is a rich source of dietary flavonoids, which have a potent antioxidative capacity [3,17-20]. Literature abounds on the anti-inflammatory actions of dietary flavonoids in general and specifically cocoa [21-23]. Given the known role of oxidative stress and inflammation in the pathophysiology of plasmodial infection and its associated damage to liver tissues [24] vis-à-vis the antioxidative and anti-inflammatory benefit of cocoa, this study compared hepatocyte ultrastructural integrity in rodent malaria treated with aqueous natural cocoa powder (NCP) ingestion with and without artemether-lumefantrine (AL). Findings will contribute to elucidating the mechanism(s) undergirding anecdotal elimination of clinical malaria in people who drink NCP daily as a beverage and offer empirical support for NCP as a diet-mediated antimalarial prophylaxis [3]. Moreover, clues will be offered to understand the mechanism(s) by which prandial NCP affords hepatoprotective benefit in rodent malaria [18,19] and alcoholic toxicity [20].

MATERIALS AND METHODS

Study design, participants and samples

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

Animals and parasites

Twenty-four male Sprague Dawley rats aged 6 - 8 weeks and weighing between 180 g - 200 g were kept under laboratory conditions with an ambient temperature of 28°C, relative humidity of 70% ± 4%, 12-hour light and dark cycle and 24-hour natural ventilation at the University of Ghana, Medical School Animal House, Korle Bu, Accra, Ghana. The rats were kept in four different cages with dimensions of 30 cm x 22 cm x 16 cm (length x breadth x height, respectively) after 7-day acclimatisation from the animal holding unit. The animals were fed 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 NMIMR, Ghana. The parasites in (infected) rat erythrocytes at a concentration of 1 x 10^5 were suspended in sodium citrate and stored in liquid nitrogen.

Experimental protocol

The 24 rats were randomly assigned by lottery to four (4) groups of six (6) animals in four cages. Once weekly, before and during the experiment, the body weights of rats were recorded. Regardless of group, each rat in the study was inoculated with 0.2 mL of parasitised blood containing 1 x 10^5 *P. berghei* (NK 65) parasites per µl of blood. Rats in group 1 (G1) were given free access to and voluntarily drank 2% (weight/volume) aqueous NCP via feeding bottle ad libitum for 14 days. Rats in group 2 (G2) similarly had access to and freely drank 2% (weight/volume) aqueous NCP ad libitum for 14 days. G2 rats were additionally administered 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 not given NCP but water ad libitum for 14 days, and the same dose of AL was given on the same days as for G2 rats. To harmonise stress associated with oral gavage, G1 rats were also given NCP via this route besides their voluntary drinking on the days that the AL oral gavage was administered to G2 and G3. Rats in group 4 (G4) served as negative control and were inoculated with the same concentration and volume of parasites but were neither given NCP nor AL. Rats from each group were coded with picric acid on specific body parts for easy identification. The treatment lasted for 14 days, during which all rats were fed the same standard.

Preparation of 2%(w/v) unsweetened NCP

NCP was prepared as previously described [19] with 2 g of commercially obtained NCP (GoodFood®, KEL Kakawa Co. Ltd. Ghana, batch no. DA1402A) dissolved in 100 ml of freshly boiled tap water. The mixture was stirred with appropriate vigour until it began to froth, indicating a uniform suspension. It was then cooled under running tap water to the ambient temperature of 28ºC. Fresh NCP suspension was prepared daily throughout the duration of the experiment, and G1 and G2 rats drank volitionally via water bottles for seven days before inoculation of parasitised RBC and 14 days post-inoculation. Administration of 0.5 mL – 0.8 mL NCP by oral gavage (per body weight of each rat) was done to equalise the stress to rats given AL by gavage and was given on the same days as AL administration.

Inoculation procedures and parasite counting

Cryopreserved parasites were taken through routine procedures to prepare an inoculum in a complete parasite medium (CPM) (Gibco, USA). After inoculation of the stock (donor) with parasites, a series of passages were run in subsequent donor rats in order to establish infection. The establishment of infection was confirmed by examination of thick and thin blood films prepared from the tail veins of

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the rats from which parasite density and percentage of parasite were calculated. Parasite density per µl of blood was determined by counting parasites against the total WBCs (~200 WBCs) counted in Giemsa-stained thick blood films, and the figure was multiplied by 8,000 (the standard WBC count per µl of blood). The calculation was done with the following equation: Parasite density = (number of parasites counted x 8000 WBCs) ÷ 200 WBCs counted. Parasitaemia was monitored every two days post-inoculation by Giemsa-stained thin blood films from tail veins, which were expressed as a percentage. Between 500 and 1000 RBCs were counted per slide with a mechanical hand tally counter (H-104, USA), and percentage parasitaemia was calculated as follows: Parasite (%) = (number of infected RBCs ÷ total number of RBCs counted) × 100.

After the desired parasite density and percentage of parasite was achieved (≥ 45%), a hypodermic needle containing 0.2 ml of trisodium citrate was used to draw blood directly from the rat by cardiac puncture using the xiphoid process as a guide. The blood was then put into Eppendorf tubes (Reagiergefab, Sarstedt Aktiengesellschaft and Co., Germany) containing 1.5 ml of normal saline. The diluted blood was transferred into a 15 ml falcon tube (Rohrchen Greiner bio-one, Germany) containing 2.0 ml of tri-sodium citrate to prevent clotting while inoculation was done. The rats were individually inoculated intraperitoneally (i.p.) with 0.2 ml of the diluted parasitised blood containing 1 x 10^5 *Plasmodium berghei* (NK65) parasites per µl of blood.

Artemether lumefantrine (AL) administration

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

Preparation of rats for liver harvesting and systematic uniform random sampling (SURS)

All animals were sacrificed on day 14 post-inoculation. Each animal was euthanised 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 a blood-given set. The right atrium was punctured to allow effluent flow. Perfusion was started with normal saline until the liver turned pale, followed by a fresh fixative (2% formaldehyde and 2% glutaraldehyde buffered at pH 7.4 with 0.1M cacodylate). Adequacy of perfusion was determined if the liver was firm when touched with a pair of forceps. Following perfusion-fixation, the liver of each rat was excised in whole from the animal and separated into the right, left, median, and caudate lobes. Using a disposable microtome blade, each lobe was sliced at a thickness of 1.0 mm^3. 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 smallest 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 (Figure 1).

Processing for transmission electron microscopy

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. Each liver slice was further cut into 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). 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) was used to systematically randomise 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). The developed films were later scanned with 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).

Hepatic ultra-structural variables (Kupffer cells, hepatic microvilli, sinusoidal diameter, and endothelial wall thickness) as indicators of liver injury

A design-based stereological system, i.e., the test system for grid-point counting (25) (Figure. 2), was used to estimate the volume density (Vv) of Kupffer cells, whilst the test frame for counting profiles (20) (Figure 3) was employed to count microvilli in the perisinusoidal spaces (of Disse). The 10 mm^2 counting frames with a test area of 58 mm x 80 mm and a total test point (Pt) of 4,640 were computer generated by Adobe Photoshop CS6 extended version and were superimposed on the micrographs. The number of points hitting the Kupffer cells was recorded as partial points (Pp). The ratio of Pp and Pt was expressed in percentage to give the volume density (Vv) of Kupffer cells. The mathematical expression of the volume density of Kupffer cells is described by the equation: Vv = Pp / Pt where: Vv = volume density, Pp = partial points and Pt = total test points.

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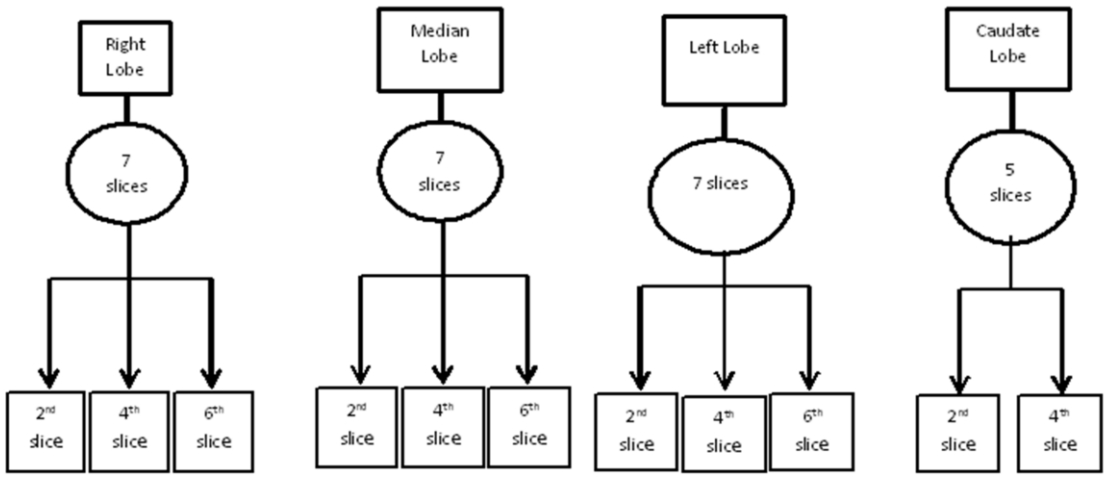

Figure 1. A chart illustrating how a rigorous systematic uniform random sampling (SURS) of liver lobes was performed in this study

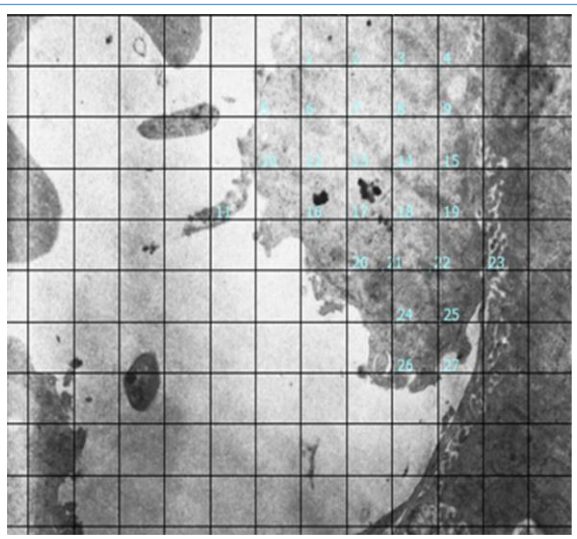

Figure 2. A transmission electron micrograph of rat liver superimposed with a lattice test grid. The numbered points (in light blue) are hitting a Kupffer cell. The stain is uranyl acetate. The bar represents 1µm.

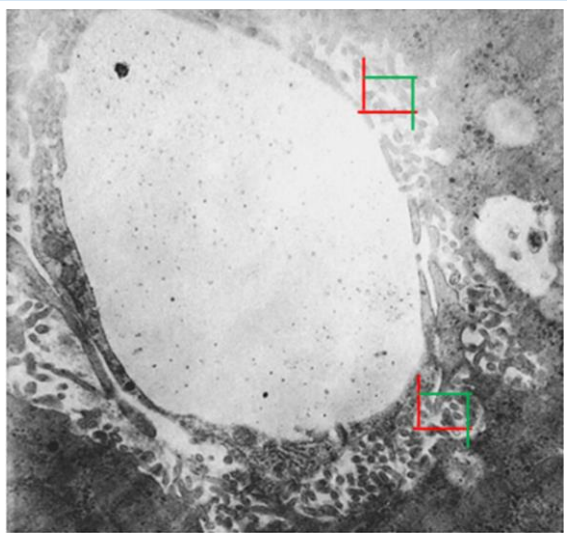

Figure 3. A transmission electron micrograph of a rat liver superimposed with a test frame for counting profiles. Hepatic microvilli are seen as extensions or profiles within the perisinusoidal spaces (of Disse) where two test frames are positioned. Stain: uranyl acetate. Bar represents 1µm.

The test frame for counting the peri-sinusoidal microvilli had an accepted line and a forbidden line. Microvilli, which hit the forbidden lines, were excluded from the counts. Counting was done by clicking the points of interest (i.e., microvilli) with the left button of the computer mouse, and the total counts were automatically calculated using the Photoshop counting tool. The Photoshop ruler tool was used to measure the short axes of sinusoidal diameters and endothelial wall thickness.

Biochemical assays

Between 10 ml to 15 ml of blood was collected transcardially from each rat using a 20 ml hypodermic syringe attached with a 21-gauge needle (BDH, England) after anaesthesia but before perfusion fixation. The blood

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was put in serum separator tubes and centrifuged at 10,000 rpm for 10 minutes. Activities of serum alanine transaminase (ALT) and serum aspartate transaminase (AST) were assayed with an automated biochemical analyser (Flexor Lab E, VITA Scientific, Netherlands) at NMIMR, Legon Ghana.

Superoxide dismutase (SOD) and Glutathione (GSH)

SOD was measured using a diagnostic kit from Cayman Chemicals, USA. A volume of 10 ml of blood was collected by cardiac puncture with a 21-gauge hypodermic needle without any anticoagulants for the SOD and GSH analysis. The blood was allowed to clot for 30 minutes at 25 ºC and centrifuged at 2,000 rpm for 15 minutes at 4ºC. Serum was diluted at a ratio of 1:5 with sample buffer before assaying for SOD using the manufacturer's standard assay procedure (Cayman Chemicals, USA). GSH level was measured using a diagnostic kit from Cayman Chemicals, USA. Five (5) ml of the blood collected by cardiac puncture was deproteinated by dissolving in 5 g of metaphosphoric acid (MPA) (Sigma-Aldrich 239275) in 50 mL water. Subsequently, an equal volume (5 ml) of the MPA reagent was added to the sample and mixed by vortexing, and the mixture was allowed to stand at room temperature for 5 minutes and centrifuged at greater than 2,000 g for 2 minutes. The supernatant was carefully collected without disturbing the precipitate. A 4 M solution of triethanolamine (TEAM) was prepared in water by mixing 531 µl of triethanolamine with 469 µl of distilled water. Fifty (50) µl of TEAM was added per ml of the supernatant and vortexed immediately. All dilutions necessary at this stage were done with *N-morpholinoethanesulphonic* acid (MES) Buffer and assayed for total GSH by following the standard assay procedure provided by the manufacturer.

Statistical analysis

GraphPad Prism version 7.0 was used to analyse the data obtained from the study. After Bartlett's test for equal variance was done, a one-way analysis of variance (ANOVA) and Kruskal-Wallis tests were performed, followed by Bonferroni and Dunn's post hoc tests, respectively, to distinguish group(s) difference(s). All data were expressed as mean ± standard deviation (SD); P values < 0.05 were considered significant.

RESULTS

Two rats from G1 and a rat each from G2, G3 and G4 died during the study. The cause of death was not determined owing to the unavailability of a veterinary pathologist. Blood from the tail vein was used to prepare thin films for parasite counting. Parasitaemia was monitored from days 4 - 8 after animals were inoculated with parasites, as presented in Figure 4. ANOVA yielded a significant difference ($p < 0.0001$), and post hoc Bonferroni multiple comparison tests also showed significant differences between the groups (Table 1).

Hepatic sinusoidal diameters

A bar chart of the mean capillary sinusoidal diameters (µm) is presented in Figure 5(a). The values obtained were G1: 80.76 µm (SD 31.63), G2: 85.75 µm (SD 34.05), G3: 105.7 µm (SD 36.67), and G4: 118.7 µm (SD 76.19). Analysis of variance yielded a significant difference ($p < 0.0001$). Post hoc analysis confirmed the significant differences between the groups, except between G1 and G2 and G3 and G4. (Table 2).

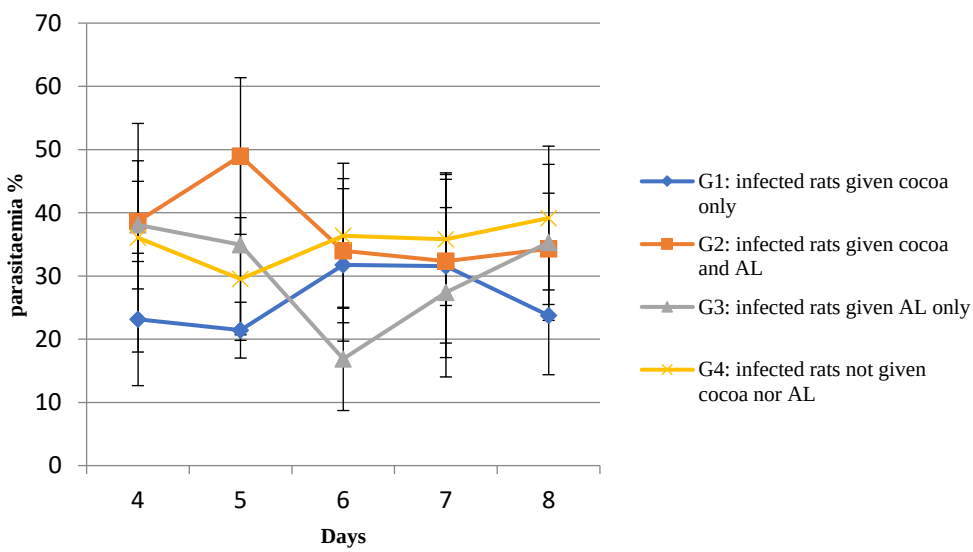

Figure 4. A line plot of trend of parasitaemia of parasitized RBCs in the infected rat groups for the from days 4 tofirst 8 days after parasite inoculation

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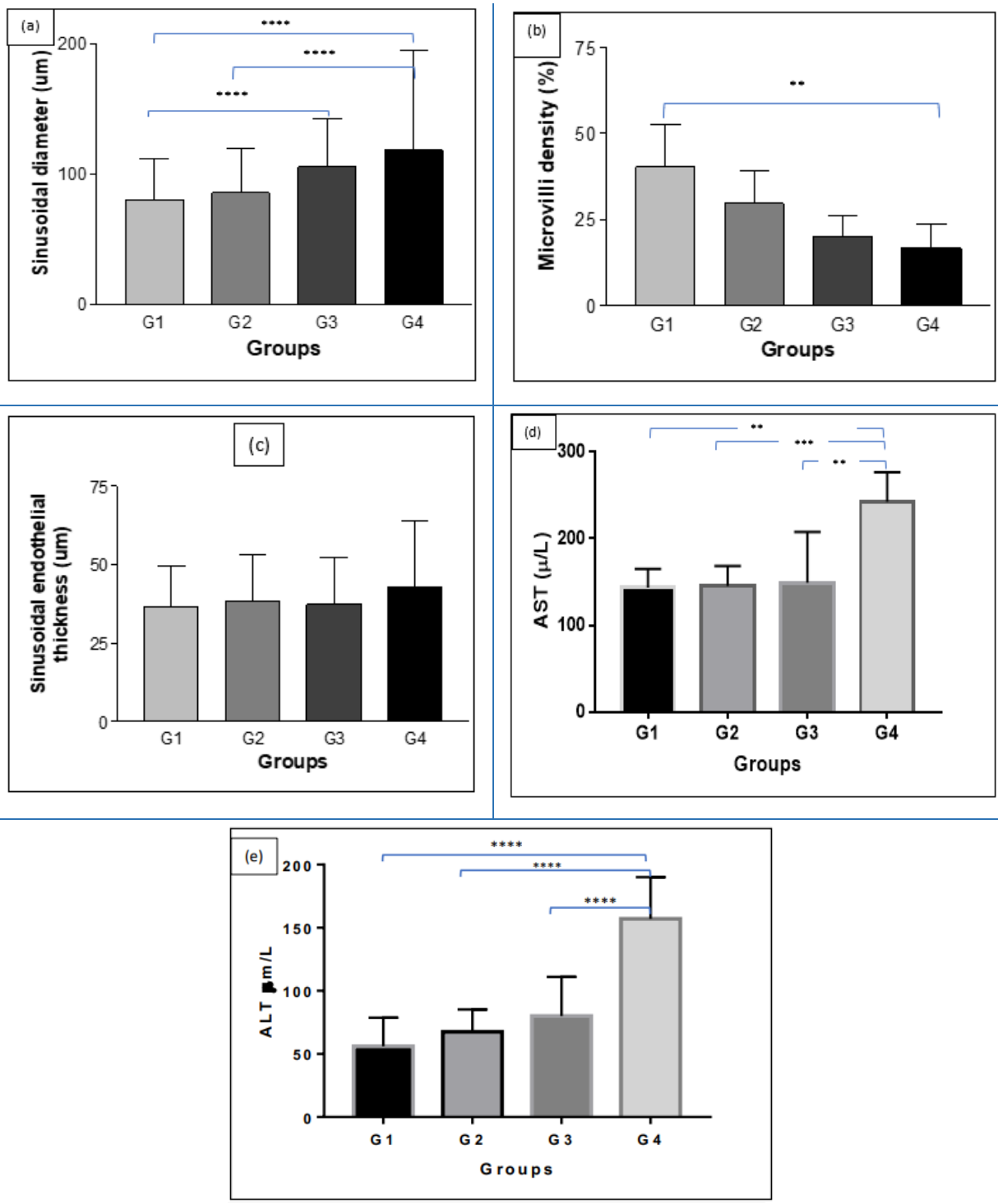

Figure 5 (a – e). Bar charts showing
(a) hepatic sinusoidal diameters (µm) in rats.
(b) Hepatic microvilli density (%) (within perisinusoidal spaces).
(c) Hepatic sinusoidal endothelial thickness (µm).
(d) Levels of AST (U/L) activity in rats after plasmodial inoculation.
(e) Levels of ALT (U/L) activity in rats after plasmodial inoculation.
Key: G1: infected rats given cocoa only, G2: infected rats given cocoa and AL, G3: infected rats given AL only, G4: infected rats neither given cocoa nor AL. Error bars represent standard deviation (SD). ** ($p < 0.005$); *** ($p < 0.001$); **** ($p < 0.0001$)

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

Groups	T	95% CI	P - value
G1 D4 vs. G3 D4*	3.703	-29.81 to -0.1059	0.0452
G1 D5 vs. G2 D5***	6.417	-43.33 to -11.76	<0.0001
G2 D5 vs. G4 D5***	5.892	7.314 to 31.59	<0.0001
G2 D5 vs. G3 D5**	4.167	1.651 to 26.45	0.007
G1 D6 vs. G3 D6**	4.519	2.771 to 26.97	0.0015
G2 D6 vs. G3 D6***	5.74	6.151 to 28.08	<0.0001
G4 D6 vs. G3 D6***	6.782	8.915 to 30.03	<0.0001

Table 2. Synopsis of Bonferroni's post hoc comparisons of sinusoidal diameters (µm) of the liver in the rat groups.

Groups	Mean Diff.	t	P - value	95% CI
G1 vs G2	-4.988	1.019	p > 0.05	-17.94 to 7.964
G1 vs G3	-24.93	5.435	p < 0.001	-37.08 to -12.79
G1 vs G4	-37.89	7.38	p < 0.001	-51.47 to -24.30
G2 vs G3	-19.95	3.364	p < 0.01	-35.64 to -4.253
G2 vs G4	-32.9	5.173	p < 0.001	-49.73 to -16.06
G3 vs G4	-12.95	2.114	p > 0.05	-29.17 to 3.267

Density of hepatic microvilli within perisinusoidal space

The mean volume density of apical hepatocyte projections into perisinusoidal spaces in the liver of rats was G1: 40.25 % (SD 12.55), G2: 29.67% (SD 9.61), G3: 20.00 % (SD 6.0) and G4: 16.57% (SD 6.95) Figure 5(b). A one-way ANOVA gave a significant value, but Bonferroni's post hoc tests showed a significant difference only between G1 and G4 (Mean difference 23.68, $t = 4.27$, $p < 0.01$, 95% CI = 6.44 to 40.92).

Thickness of sinusoidal endothelium

A Bartlett's test for equal variance indicated that data on sinusoidal endothelial thickness failed the normality test. Hence, a non-parametric test of ranks was used to compare values from the four groups of rats. Figure 5(c) shows a bar chart showing the mean thickness of the endothelium of sinusoids. The Kruskal-Wallis test for the medians of sinusoidal endothelial thickness (in µm) yielded a significant value ($p < 0.05$). Dunn's post hoc test produced only two significant differences. (i) Between G1 and G4 (difference in rank sum = -19.88; $p < 0.05$), and (ii) Between G3 and G4 (difference in rank sum = -20.89; $p < 0.05$).

Volume density of Kupffer cells

The respective median volume densities of Kupffer cells were 0.55%, 0.70%, 0.80%, and 1.00 % for G1, G2, G3, and G4 rats. The percentages of mean volume densities of Kupffer cells in the liver of the animals were G1 (0.55, SD 0.35), G2 (0.67, SD 0.58), G3 (0.83, SD 0.15), and G4 (0.97, SD 0.15). The values were not significantly different among the groups (F Statistic: 2.663; Kruskal Wallis value: 5.829; both with $p > 0.05$).

Serum GSH concentration and levels of SOD activity

The mean serum GSH concentrations (µM) measured were 1.83 (SD 0.28), 1.79 (SD 0.12), 1.73 (SD 0.16), and 1.64 (SD 0.24) for G1, G2, G3, and G4 rats, respectively. The mean serum SOD activity assayed in the rats was as follows. G1 (1.23, SD 0.42); G2 (1.26. SD 0.43); G3 (1.22, SD 0.41), and G4 (1.12, SD 0.41). One-way ANOVA on the mean serum GSH concentration (F value 0.7) and SOD activity (F value 0.09) did not yield significant differences ($p > 0.05$).

Biochemical markers of liver function

Serum AST values obtained from the rats at the termination of treatment are presented in Figure 5(d). A one-way ANOVA on serum AST showed significant differences between the four groups ($p < 0.05$). Bonferroni's post hoc tests confirmed statistical differences between the following treatment groups. G1 and G4 (mean difference = -99.03; $t = 3.94$; $p = 0.004$; 95% CI = -171.9 to -26.130, G2 and G4 (mean difference = -97.23; $t = 4.53$; $p = 0.001$; 95% CI = -159.4 to -35.06), as well as G3 and G4 (mean difference = -93.33; $t = 4.49$; $p = 0.001$; 95% CI = -153.5 to -33.13). However, no significant differences in serum AST existed between G1 and G2, G1 and G3, or G2 and G3. Serum ALT for the four rat groups is presented in Figure 5(e). A one-way ANOVA and Bonferroni's post hoc comparisons of serum ALT values in the rats produced statistics similar to those obtained for AST analysis. No significant differences were found between ALT values for rats in G1 vs. G2, G1 vs. G3, or G2 vs. G3. With a mean difference of 101.10, a t-value of 6.92 at $p < 0.0001$, and a 95% confidence interval of -142.7 to -59.53, the ALT was significantly lower in G1 compared to G4 rats. Comparably, ALT was lower in G2

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than in G4 rats (mean difference = -89.50; t = 6.92; p < 0.0001; 95% CI = -126.3 to -52.65). Group 3 rats had statistically depressed ALT levels compared to Group 4 rats (mean difference = -77.09; t = 5.67; p < 0.0001; 95% CI = -115.8 to -38.40).

DISCUSSION

In a previous study [19], we demonstrated at the light microscope level that hepatic damage was attenuated in mice infected with *P. berghei* when given free 24-hour access to 2% NCP. The present study extended the previous by comparing oral gavage administration of a therapeutic dose of AL with voluntary ingestion of 2% NCP and assessing morphological variables of hepatic damage at the ultrastructural level. Notably, NCP administration commenced seven days before plasmodium infection and continued throughout the 14 days of the experiment. The afforded hepatoprotection by NCP administration in the present study, therefore, reinforces its prophylactic efficacy [3]. To facilitate the explanation of liver tissue damage, percentage parasitemia was assessed daily from the 4th to the 8th day after animals were infected but was curtailed for the last six days of the study to forestall possible complications of results by blood loss through the daily sampling. The results of this study suggest that NCP conferred hepatoprotection not because of reduced parasitaemia since, by day 8, there was no difference in parasite load among the rat groups (Figure 4). It is apparent that whereas AL alone (G3 rats) significantly depressed parasitaemia on day 6, it increased steadily by day 8, possibly as a result of the waning of the efficacy of AL after the last shot on day five post-inoculation. AL combination with NCP did not show any significant difference since parasite load in G2 rats was not different from that of G1.

The parasite load in the untreated group (G4) was low on day five but then shot up steadily for the subsequent days up to day eight because there was no intervention (Figure 4). It is noteworthy, moreover, that in all other variables assessed in the present study, co-administration of AL/NCP (G2) consistently produced worse statistical values than NCP only (G1) but better results (albeit not always significant) than control (G4) and AL alone (G3). This finding is interpreted to mean that prophylactic use of NCP was better for preventing hepatic tissue damage in plasmodial infection. Given the mutually reinforcing roles of oxidative stress and inflammation in malaria pathophysiology [8], serum GSH and SOD, as well as AST and ALT, were assessed at the end of the experiment. AST and ALT are liver enzymes that reveal tissue damage and are elevated in various disease conditions, including severe/complicated [14] and uncomplicated malaria [26,27]. These liver enzymes also directly correlate with the inflammatory markers C-reactive protein (C-RP) [28,29]. The trends of serum antioxidants (GSH and SOD) measured in the present study were G1>G2>G3>G4 and G2>G1>G3>G4, respectively. It is apparent that the 14-day duration of this study could have been too short to elicit significant differences, as other studies recorded high antioxidant capacity when cocoa was added to the diet of humans and rats [30-35]. It is worth mentioning that other researchers in our laboratory who experimented over four weeks or longer registered significant serum antioxidant levels in animals given NCP [36,37].

It is interesting that in the present study, the trends of serum AST and ALT were G1<G2<G3<G4. The marginally higher levels of these enzymes in animals given AL-only may be explicable by the report that treatment with AL causes a harmless self-limiting rise in liver enzymes [38]. However, statistically lower AST and ALT in other rat groups compared with G4 rats suggest that treatment of rats with NCP only, NCP & AL, and AL-only did reduce inflammatory pressure exerted by plasmodium parasitaemia. Elevated AST levels reflect liver damage [39], and they are the most sensitive markers employed in the diagnosis of hepatic damage because they are cytoplasmic enzymes released into circulation after cellular damage [40]. Since plasma clearance of AST is modulated by the activity of sinusoidal liver cells, during progressive fibrosis and cirrhosis, the functions of these cells are progressively impaired, resulting in a relative increase in AST levels [41]. The present study focused on four ultrastructural indicators of liver tissue damage, namely, increased sinusoidal diameter, decreased density of microvilli in perisinusoidal space (of Disse), increased sinusoidal endothelial thickness, and increased volume density of Kupfer cells. According to these criteria, our findings were in order of mitigated hepatic damage: G1>G2>G3>G4. Animals given NCP only (G1) consistently had significantly less damaged liver than control (G4) and G3 rats according to sinusoidal diameter. Whereas rats given NCP & AL (G2) had statistically less damaged liver tissue than control (G4) and AL-only treated rats (G3) with respect to sinusoidal diameter, rats given only AL (G3) had less damaged liver than control (G4) based on sinusoidal endothelial thickness. Moreover, better attenuation of inflammation arising from the malaria infection by cocoa flavonoids [42,43], as evidenced by lower serum AST/ALT values discussed above, suggests better overall liver protection.

No differences were found in this study in terms of the volume density of Kupffer cells, which may have resulted from limitations of the stereological method used or the fact that parasitaemia did not reach the severe stage [24] that is characterised by increased numbers of these phagocytic cells. A noteworthy limitation of this study is our inability to determine the reason for the animal deaths recorded in all groups.

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Conclusion

Evidence has been produced to show that damage to the ultrastructural liver morphology in murine malaria was significantly mitigated by daily ingestion of NCP compared with AL treatment with respect to hepatic sinusoidal endothelial thickness and density of hepatic microvilli despite parasitaemia being comparable to untreated control rats. Correspondingly, the relatively minimised sinusoidal distension buttressed by lower serum AST/ALT but not GSH/SOD with NCP administration provokes the thesis that the anti-inflammatory activity of cocoa flavonoids may account for observed better hepatoprotection in this study.

DECLARATIONS

Ethical consideration

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.

Consent to publish

All authors agreed on the content of the final paper.

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Competing Interest

The authors declare no conflict of interest for this paper

Author contributions

EA conceived the idea which was his Ph.D project, designed the manuscript, analysed and interpreted the data, and approved it for publication. FKA contributed to the conception and design, reviewed the draft of this manuscript, and approved the final version for publication. He was the lead supervisor of the Ph.D. project. PA contributed to the design, advised on animal experimentation and statistical analysis, and approved the final version for publication.

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Availability of data

Data is available upon request to the corresponding author.

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