

Natural cocoa ingestion reduced liver damage in mice infected with *Plasmodium berghei* (NK65)

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Purpose: This study tested whether natural cocoa powder ingestion could mitigate hepatic injury coincident with murine malaria. *Plasmodium berghei* infection causes liver damage including hepatic sinusoidal distension, and elevated serum alanine transaminase (ALT) and aspartate transaminase (AST) levels. According to literature, these pathologies largely result from activity of reactive oxygen species (ROS) and may be extenuated by antioxidants.

Animals and methods: Thirty Balb/c mice were randomly assigned to three equal groups. One of two groups of mice inoculated with 0.2 mL of *P. berghei*-parasitized red blood cells (RBCs) was given unrestricted 24-hour access to a natural cocoa powder beverage (2% by weight) in place of water. The third group of mice were neither infected nor given cocoa. All mice were fed the same standard chow. After 6 days, mice were sacrificed and their livers processed for histomorphometric assessment of mean hepatic sinusoidal diameter as a quantitative measure of altered morphology. Serum ALT and AST were measured as a gauge of functional impairment.

Results: Compared with uninfected mice, hepatic sinusoidal diameter in *P. berghei*-infected mice not given cocoa increased by 150%, whereas a smaller increase of 83% occurred in infected mice that ingested cocoa. Mean serum ALT increased by 127% in infected mice not given cocoa and 80% in infected mice that consumed cocoa, compared with the value for uninfected mice. Similarly, mean serum AST was raised by 141% in infected mice not given cocoa and 93% in infected mice that drank cocoa.

Conclusion: Distension of hepatic sinusoidal diameter in *P. berghei*-infected mice was reduced by 67%, whereas respective elevations of serum ALT and AST concentrations were reduced by 47% and 48% via ingestion of cocoa. Anti-inflammatory and antioxidant components of cocoa probably mediated the demonstrated hepatoprotective benefit by blunting pernicious ROS activity in *P. berghei*-infected mice.

Keywords: polyphenol antioxidants, murine malaria, hepatic sinusoids, reactive oxygen species

Introduction

Extensive efforts made over the last century to understand and control malaria have yet to minimize its significance as a major cause of morbidity and mortality in humans, and a number of factors have led to its presenting with unusual features.¹ In mice, significant liver ultrastructural pathology has been demonstrated in advanced stages of *Plasmodium berghei* malarial infection.² The liver is of great interest in malaria parasitemia for at least three reasons. Firstly, it is a target organ that plays a key role in the parasite's developmental cycle.³ Secondly, parasite activity combine with the

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host's immune response to give rise to chronic inflammatory insults,^{4,5} which predisposes the organ to deleterious conditions including dysfunction and fulminant hepatic failure,¹ as well as hepatocellular cancer, and non-alcoholic fatty liver disease.⁶ Thirdly, understanding of the liver stage of malaria parasites offers a promising target for antimalarial strategies that aim to establish immunity against the malaria parasite.⁷

After subcutaneous deposition by a biting female anophles mosquito, malaria sporozoites are transported to the liver via the bloodstream where they invade hepatocytes and undergo many rounds of schizogony.⁸ The parasites migrate through several hepatocytes causing cell death before eventually settling down in a final hepatocyte for multiplication and differentiation into merozoites.^{9,10} Hepatocellular damage results from the generation of free radicals produced during malaria infection.¹¹ A link between free radicals, reactive oxygen species (ROS), and oxidative stress in tissue damage is now well established. It has been shown that increased oxidative stress during malaria infections,¹² arises from both the parasite's metabolism,⁴ and the host's immune response.⁵ With respect to liver pathology, oxidative stress is one of the causes of DNA damage associated with hepatocellular carcinoma in chronic viral hepatitis;¹³ whilst ROS and lipid peroxidation products contribute to both onset and progression of hepatic fibrosis.¹⁴

The mouse has a liver with four major lobes, just as in humans,¹⁵ and has a gall bladder (which rats lack),¹⁶ making it a good model for the study being reported. Moreover, *P. berghei* (murine malaria) is one of the most widely used experimental models to study malaria transmission.¹⁷ One striking histological feature of the acute stage of malarial parasitemia is gross congestion in the sinusoids and hypertrophy of hepatic macrophages (Kupffer cells) that arises as they engulf parasitized and unparasitized red blood cells, remnants of parasites, granules and masses of hemozoin containing hemosiderin.¹⁸ Hepatic damage is also characterized by markedly elevated levels of alanine transaminase (ALT), aspartate transaminase (AST) and bilirubin, coupled with a marked hepatic oxidative stress.¹⁹

Cocoa, a product derived from the beans of the *Theobroma cacao* plant, has been consumed since 600 BC by ancient civilizations, such as the Mayans and Aztecs.²⁰ A rich source of flavonoid and theobromine, cocoa has been used for centuries as a medicine to combat inflammation, pain, and numerous other ailments.²¹ Cocoa flavanols are particularly notable for their powerful antioxidative properties, which is mainly related to their inherent capacity to scavenge free

radicals, thereby counteracting conditions of oxidative stress and coincident tissue damage.^{22,23} This antioxidant activity has been proven with isolated cocoa flavonoids, including the main compounds, catechin, epicatechin, and procyanidins;^{24,25} as well as the cocoa metabolites.²⁶ For instance, the flavonol quercetin (a cocoa metabolite) has been shown to prevent hepatotoxicity and nephrotoxicity caused by oxidative damage in rats.^{27,28} Moreover, consumption of cocoa powder enhances the antioxidant capacity of plasma, and decreases the content of lipid oxidation products in human²⁹ and rat plasma.³⁰ This study used natural cocoa because Gu et al³¹ showed that, being the least processed of consumed cocoa products, it contains the highest levels of total antioxidant capacity and procyanidins.

Material and methods

Animals

Thirty male Balb/c mice aged 6–8 weeks and of body weight 12–25 g were used. All mice were kept under the same laboratory conditions of temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$), relative humidity ($70\% \pm 4\%$), and were exposed to a 12-hour light and dark cycle, and adequate ventilation. Mice were transferred from the breeding unit to the infectious unit of the animal experimentation unit of the Noguchi Memorial Institute for Medical Research for 7 days acclimatization before commencement of the experiments. During this period their body weights were recorded and they were fed with commercially obtained standard feed from Ghana Agro Food Company (GAFCO, Tema, Ghana), and given freshly filtered tap water every morning. The study protocol was approved by the Ethical and Protocol Review Committee of the University of Ghana Medical School. Procedures involving the care and use of mice conformed to the institutional guidelines in compliance with national and international laws and guidelines for the use of animals in biomedical research.

P. berghei (NK65) was procured from the Immunology Department of the Noguchi Memorial Institute for Medical Research.

Experimental protocol

Mice were randomly assigned to three experimental groups of ten animals per group, and were separated in three different cages of dimension 30 cm \times 22 cm \times 16 cm. Mice in group 1 (G_1), were inoculated with 0.2 mL of parasitized blood containing 10^5 parasites per μL of blood and given unrestricted 24 hours access to 2% (weight/volume) aqueous natural cocoa powder in place of drinking water. Mice in group 2 (G_2), were inoculated with 0.2 mL of parasitized

blood containing 10^5 parasites per μL of blood and were not given cocoa, but had an unrestricted 24 hours access to drinking water. Mice in group 3 (G_3) represented the control group which were neither infected nor fed cocoa, but had an unrestricted 24 hours access to drinking water. Animals in all three groups were fed with commercially obtained standard feed from Ghana Agro Food Company (GAFCO) during the experiment.

Cryopreserved parasites were taken through routine procedures to prepare an inoculum in complete parasite medium. After inoculation of stock (donor) mice with parasites, a series of passages was run in order to establish infection. Blood films were prepared from donor mice to estimate parasite density and parasitemia required to achieve infection of experimental mice. Blood from the tail vein of infected mice was placed on a clean glass slide (All Pro Processed Microscope Slide, Cat #7105, Surgifriend Medicals, Middlesex, England) and taken through the routine procedure for preparation of thick and thin films and examined under a light microscope (Leica, Galen III, Cat# 317505; Leica Microsystems, Wetzlar, Germany) with immersion oil and $\times 100$ objective lens. When the required parasite density (10^5 parasitized red blood cells [RBC]) was achieved in donor mice, blood was drawn by cardiac puncture for inoculation of the experimental mice.

A hypodermic needle containing 0.2 mL of trisodium citrate (to prevent blood from clotting) was inserted into the thorax using the xiphoid process as a guide, and blood was drawn by cardiac puncture. The blood was then put into Ependorff tubes (Reagiergefah, Sarstedt AG and Co, Numbrecht-Rommelsdorf, Germany) containing 0.5 mL normal saline. The diluted blood was then transferred into a 15 mL falcon tube (Rohrchen; Greiner Bio-One International AG, Kremsmuenster, Austria) containing 2 mL of trisodium citrate. Mice in G_1 and G_2 were inoculated intraperitoneally with 0.2 mL of diluted parasitized blood containing 10^5 *P. berghei* (NK65) parasites per μL of blood.

Parasite density (of inoculum) per μL of blood was determined by counting the number of parasites against the total white blood cells (~ 200 white blood cells) in Giemsa-stained thick blood films and the result multiplied by 8000 (the standard white blood cell count per μL of blood). The calculation was done as follows:

$$\text{Parasite density } (\mu\text{L}) = \frac{\text{No of parasites}}{200 \text{ WBC}} \times 8000 \quad (1)$$

Parasitemia was monitored every 2 days post inoculation through Giemsa-stained thin blood films and expressed as per-

centages of at least 500 RBCs. Between 50–1000 RBCs were counted per slide with a mechanical hand tally counter (WJT-002A, Wenzhou Hualong Instrument & Apparatus, China) and percentage parasitemia was calculated as follows:

$$\text{Parasites \%} = \left[\frac{\text{Number of infected RBCs}}{\text{Total number of RBCs counted}} \right] \times 100 \quad (2)$$

Biochemical analysis

On day 6 post inoculation, about 0.8 mL blood was collected by cardiac puncture from each experimental animal, into a clean dry centrifuge tube. The blood sample was allowed to stand for 2 hours to clot, and then centrifuged at 10,000 rpm for 10 min using a Wisperfuge centrifuge (model 1384; GEA Westfalia Separator, Oelde, Germany). The blood clot was gently loosened from the sides of the tube with an applicator stick to facilitate serum separation. Serum was then collected from the clot by micropipette, into sterile Ependorff tubes (Reagiergefah) for the measurement of biochemical indices. Activity of serum ALT and AST (both expressed as μL) were assayed with an automated biochemical analyzer (Vitalab Flexor E; 115W \times 49H \times 56D cm; Vital Scientific, Dieren, Netherlands). Serum from individual blood samples was measured separately and average values calculated for each group of mice.

Preparation of unsweetened natural cocoa drink

Two grams of GoodFood® Natural Cocoa Powder (batch number KK1004A1; Kakawa Enterprise Ltd, Accra, Ghana) was weighed using a chemical weighing balance (P1200; Mettler-Toledo International Inc, Greifensee, Switzerland) and dissolved in 100 mL of hot water. The dissolution of cocoa powder was ensured by adequate stirring to a uniform suspension. The hot cocoa drink was cooled under running tap water to 35°C before being made available to the mice. Fresh cocoa drink was prepared every morning and G_1 mice had unrestricted access to it in place of drinking water. The drink was administered in a bottle with a tube from which the animals could suck. The bottles were washed each morning and filled with a freshly prepared drink. Mice in G_2 and G_3 were given tap water without cocoa powder. The cocoa drink in the feeding bottles was periodically shaken to prevent sedimentation of the particles of cocoa powder, which otherwise could clog the teats of the bottles and reduce the free flow of the drink when sucked by the mice. The volume of cocoa drink ingested by the mice was estimated by subtracting the final volume of the drink from the initial volume served. The composition of the cocoa powder used in this study is given in Table 1.

Table I Composition of Goodfood® Natural Cocoa Powder according to manufacturer's specification

Component	Amount (g/100 g)
Total dietary fibre	34.3
Total carbohydrates	15.5
Protein	24.0
Moisture	6.0
Free fatty acid	1.2
Theobromine	1.2
Total ash	5.7
Acid insoluble ash	0.1
Ferulic acid**	8.66–28.8
p-coumaric acid**	4.2–16.0
Gallic acid**	3.0–12.0

Notes: **Components not included in manufacturer's specification but provided by Drs Daniel Abaye and Paul Amuna of University of Greenwich, Medway Campus, Kent, England. Liquid chromatography-electrospray ionization mass spectrometry determinations of polyphenols were performed by their research student Abisola Adeyemi on Goodfood natural cocoa powder supplied to them by FKA as part of a collaborative initiative independent of the study reported in this paper.

Measurement of liver weight

All mice were sacrificed on day 6 post inoculation after first anesthetizing them with chloroform in a tight fitted jar. Freshly dissected liver was put in ice-cold physiological saline to wash off blood, and blotted dry with filter paper. The liver was then weighed (in grams) using the Mettler balance. Two weight readings were taken for each liver and the average was determined. The hepatosomatic index (HSI) was determined by dividing the liver weight of each mouse by its respective body weight at termination of experiment.

Sampling and processing of mouse liver for stereological assessment

Mice livers were fixed in 10% (by volume) buffered formalin solution for 7 days, and sampled using a stepwise systematic random sampling technique. The liver of each mouse was separated into the right, left, median, and caudate lobes and each lobe cut with a disposable microtome blade, into 2 mm thick slices. Four slices each were obtained from the right, left and median lobes, whilst two slices were obtained from the caudate lobe because it was the smallest of all the lobes. A slice was selected at random to represent each of the four lobes of the liver of each mouse, for routine histological processing. In all, a total of 120 liver slices were processed for microscopic examination. Paraffin wax blocks of liver tissues were prepared using a fully automated tissue processor (Leica Tp 1020; Leica Microsystems; Ernst-Leitz-Strasse, Wetzlar, Germany) and tissue blocks were cast using a molten wax dispenser, plastic cassettes and stainless steel mould boxes. Sectioning of blocked tissues was done using a rotary microtome (Leica 2235; Leica Microsystems) at

5 µm thickness. The sections were stained with hematoxylin and eosin using an automatic stainer (Leica Autostainer XL; Leica Microsystems; Ernst-Leitz-Strasse, Wetzlar, Germany). To identify the slides, the code of each mouse was written on the frosted sides of the slides.

Measurement of sinusoidal diameters

A design-based stereological assessment was conducted to determine the diameter of the liver sinusoids, as a quantitative measure of altered liver morphology. Five slides of liver tissues out of ten per mouse were randomly sampled for the morphometry. Twenty microscopic fields were randomly captured from each of the selected slides onto a computer equipped with Microsoft Publisher software (Publisher software version 2007, Microsoft, Redmond, Wash), using a digital eye piece (Catalogue # MA 88;C & A Scientific Co Inc, Manassas, Virginia) attached to a light microscope (Leica Galen III). Microscope field sampling was randomized manually using the X and Y axes of the microscope stage micrometer. This was done by alternately moving three graduations on the X-axis and subsequently three graduations on the Y-axis. When at any point, a tissue field was in focus, the image was captured and saved on the computer. This was done until 20 micrographs were obtained from each slide. Two hundred micrographs were obtained from each experimental group. A microscope stage graticule (Graticules Ltd, Kent, UK) was photographed at the same microscopic magnification as the micrographs of liver and used to calibrate a ruler, which was in turn used to measure the short diameter of sinusoids. Ten sinusoids were randomly measured per micrograph. In all, a total of three thousand sinusoidal diameters were measured.

Statistical analysis

All results were expressed as arithmetic mean and standard deviation (SD). Statistical significance of the difference between group means was performed by Student's *t* test and one-way analysis of variance (ANOVA). Differences with $P < 0.05$ were considered to be significant. SPSS software (version 16.0; SPSS Inc, Chicago, IL) was used for these analyses. Further analysis of percentage RBC parasitemia was performed using GraphPad Prism software (version 3.0; GraphPad, La Jolla, CA).

Results

Each mouse in the control (G_3) and in infected mice not given cocoa (G_2) groups took on a daily basis, an average of 13.86 mL (SD 1.07) and 10.71 mL (SD 4.50) of water, respectively. Each infected mouse in the group given cocoa (G_1)

consumed on daily basis an average of 10.48 (SD 3.24) mL of natural cocoa drink. ANOVA showed that these volumes of fluid consumed by the three groups of mice were not significantly different.

Significantly increasing RBC parasitemia occurred in infected mice (Table 2) over the six days of the study, both in animals that drank cocoa and those that did not. Parasitized RBCs and liver macrophages (Kupffer cells) that had phagocytosed infected cells were observable in the central veins of liver sections of infected mice (Figure 1). Bonferroni post-hoc tests affirmed increasing parasitemia over the three days of blood sampling in *P. berghei*-infected mice regardless of whether they drank cocoa or not, except between days two and four in animals that did not drink any cocoa (Table 3). Whether or not mice drank cocoa failed to exert statistical main effect or interaction on percentage parasitized RBCs (Table 4) in two-way ANOVA comparisons with cocoa intake and days of parasitemia as the variables. In agreement with data in Table 2, the two-way ANOVA produced significant main effect of days of parasitemia (Table 3). However, post-hoc tests showed nonsignificant differences between percentage RBC parasitemia in mice that drank cocoa and those that did not, when data were compared according to corresponding number of days after parasite inoculation (Table 5). Indeed, the only fatality recorded in this study was a rodent that drank cocoa, albeit it died before day four after inoculation with parasitized RBCs at the time when parasitemia was relatively low (Table 2); absence of veterinary post mortem service did not permit establishment of the cause of death.

Before they were inoculated with parasitized RBCs, mean body weight of infected mice given cocoa was 24.11 g (SD 1.85) and this decreased to 20.77 g (SD 2.23) after 6 days of infection. Similarly mean body weight of infected mice not given cocoa was 22.90 g (SD 1.79) before their inoculation and 20.34 g (SD 1.83) after 6 days infection. Control mice had an average body weight of 22.35 g (SD 1.62) at commencement and 22.24 g (SD 1.41) after the 6 days (of

experimentation with infected mice). ANOVA yielded a nonsignificant F-statistic (2.48, $P = 0.10$, $DF = 2$) for mean baseline body weight and body weight after 6 days of infection ($F = 2.93$, $P = 0.07$, $df = 2$). The respective mean liver weights on day 6 after infection were 1.29 g (SD 0.15) for infected mice given cocoa, 1.32 g (SD 0.16) for infected mice not given cocoa, and 1.27 g (SD 0.16) for control mice. One-way ANOVA indicated no statistical differences among the groups ($P = 0.77$). The mean HSI obtained in the three groups of mice on day 6 after infection was 6.21 (SD 0.35) for infected mice given cocoa, 6.49 (SD 0.53) for infected mice not given cocoa, and 5.76 (SD 1.03) for control mice. Again, one-way ANOVA showed nonsignificant difference ($P = 0.08$) among the groups.

Liver histoarchitecture from a *P. berghei*-infected mouse given and another not given natural cocoa to drink, as well as an uninfected control mouse, are all illustrated in Figure 2. The mean hepatic sinusoidal diameter (MHSD) for infected mice given cocoa was 15.22 μm (SD 5.42), for infected mice not given cocoa was 20.80 μm (SD 6.08), and for control mice was 8.31 μm (SD 2.64). One-way ANOVA gave a significant F-statistic of 844.22 with $P = 0.001$ and $DF = 2$. Bonferroni post-hoc multiple comparison tests yielded significant differences ($P < 0.001$) among all three groups. Thus, infected mice that were not given cocoa had the highest and control mice the lowest MHSD. Infected mice given cocoa had an intermediate MHSD that was significantly higher than the value for control mice and significantly lower than the value for infected mice not given cocoa.

The average serum ALT (in μL) in *P. berghei*-infected mice given cocoa, infected mice not given cocoa, and control mice were 67.18 (SD 16.50), 84.38 (SD 28.74), and 37.23 (SD 7.08), respectively. One-way ANOVA yielded significant F-statistic of 5.95 ($P = 0.023$, $DF = 2$). Bonferroni post-hoc multiple comparison test was nonsignificant ($P = 0.18$) between infected mice given cocoa and control mice; significant ($P = 0.02$) between infected mice not given cocoa and control

Table 2 Means, and comparative statistics on parasitized RBCs coincident with or without cocoa ingestion in *Plasmodium berghei*-infected mice

Animal group and Variable/parameter	Mice given free access to cocoa drink			Mice not given access to cocoa drink		
	2	4	6	2	4	6
Number of days after parasite inoculation						
Number of animals (n)	10	9	9	10	10	10
Percentage parasitized RBCs	1.726	13.446	27.627	5.190	11.864	35.451
Standard deviation (SD)	1.051	6.475	15.377	1.984	4.589	15.429
Repeated measures ANOVA	F value = 20.31; $P < 0.0001$			F value = 40.65; $P < 0.0001$		

Abbreviations: RBC, red blood cells; ANOVA, analysis of variance.

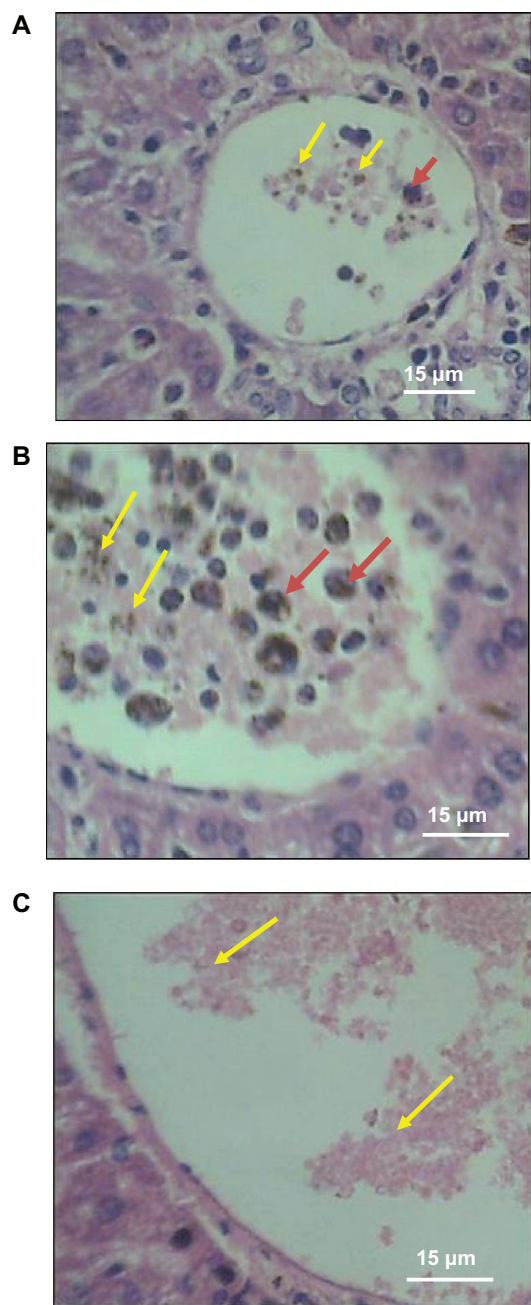


Figure 1 H&E stained liver sections showing profiles of central veins of (A) infected mouse given cocoa and (B) infected mouse not given cocoa containing parasitized RBCs (yellow arrows) and phagocytosed parasitized RBCs (red arrows). Central vein of control mouse (C) contain unparasitized RBCs (yellow arrows).

mice, and nonsignificant ($P = 0.74$) between infected mice given cocoa and infected mice not given cocoa.

Average AST (in μL) was 257.30 (SD 74.99) for infected mice given cocoa, 320.60 (SD 68.30) for infected mice not given cocoa, and 133.08 (SD 65.75) for control mice. One-way ANOVA gave significant F-statistic of 7.47 ($P = 0.012$; $\text{DF} = 2$). Post-hoc multiple comparison test was nonsignificant ($P = 0.10$) between infected mice given cocoa and control mice,

significant ($P = 0.01$) between infected mice not given cocoa and control mice, and nonsignificant ($P = 0.70$) between infected mice given cocoa and infected mice not given cocoa.

Discussion

This study was undertaken to ascertain the hepatoprotective activity of cocoa in reducing damage caused by plasmodial malaria. HSI was measured as a proxy for injurious activity of malaria infection in this study. Although the trend indicated that infected mice given cocoa had intermediate value between higher HSI for infected mice not given cocoa and lower HSI for control mice, these differences were not significant.

This study has confirmed the power of MHSD to distinguish the extent of damage caused to the liver by *P. berghei* infection. Probably for the first time, this study has shown that voluntary consumption of natural cocoa by mice reduced sinusoidal distension coincident with parasitemia by 67% compared with controls and infected mice that did not drink cocoa. Voluntary ingestion of cocoa was preferred to other routes of administration because it better reflected the typical route of administration that would be used by humans who were encouraged to drink natural cocoa as antimalarial prophylaxis.³² The finding in this study that MHSD was higher in the infected mice (G_1 and G_2) than in control mice, is consistent with previous work that also showed inoculation of mice with *P. berghei* resulted in liver damage evidenced by distension of hepatic sinusoids.^{33,34} Evidence of hepatic damage in the present study included disruption of the trabecular structure of hepatocytes due to dilatation of hepatic sinusoids, as well as hypertrophy of Kupffer cells, also previously reported.¹⁸

A direct in vitro inhibitory activity of extract and fractions of natural cocoa powder against *P. falciparum* has already been demonstrated,³⁵ so the hepatoprotective effect of cocoa in the present study suggests that prophylactic antimalarial benefit of regular ingestion of natural cocoa would take multiple forms. With respect to possible mechanism(s) of action of natural cocoa in mitigating the damage to liver that accompanies plasmodial infection, it is attractive to speculate that the notable antioxidative^{24–26} and antiinflammatory³⁶ properties of dietary flavanols play a key role. For instance it has been noted that enhanced glutathione release by liver parenchymal cells traps ROS generated by Kupffer cells and neutrophils in the vasculature, thereby attenuating liver injury.³⁷ It has also been observed that apoptosis of endothelial cells caused by severe malaria could be reduced by antioxidants.^{38,39} Furthermore, the flavonoid quercetin is reported to be capable of preventing hepatotoxicity caused by oxidative damage.^{27,28} Following from these, it is postulated that in the current study, components in

Table 3 Bonferroni's post hoc statistical analyses of percentage parasitized RBCs coincident with or without cocoa ingestion in *Plasmodium berghei* infected mice within the groups

Variable/parameter	Mice given free access to cocoa drink			Mice not given access to cocoa drink		
Number of days after parasite inoculation	TWO ^a	FOUR ^b	SIX ^c	TWO ^d	FOUR ^e	SIX ^h
Bonferroni's Multiple Comparison Test –t value	3.035	6.707	3.484	1.893	8.581	6.688
Probability (P) sampling days compared	^{ab} P < 0.05	^{ac} P < 0.001	^{bc} P < 0.01	^{de} P > 0.05	^{dh} P < 0.001	^{eh} P < 0.001
95% confidence interval of the difference	–22.04 to –1.397	–36.22 to 15.58	–25.06 to –3.301	–15.98 to 2.633	–39.57 to –20.95	–32.89 to –14.28
Degrees of Freedom (DF)	DF for Group = 2, DF for Individual = 8			DF for Group = 2, DF for Individual = 9		

Notes: ^{ab}comparison between mean percentage parasitized RBCs in blood samples taken on days two and four after *P. berghei* inoculation in mice given cocoa drink. ^{ac}comparison between mean percentage parasitized RBCs in blood samples taken from mice given cocoa drink on days two and six. ^{bc}comparison of mean percentage parasitized RBCs in blood samples taken from mice given cocoa drink on days four and six. ^{de}comparison between mean percentage parasitized RBCs in blood samples taken on days two and four after *P. berghei* inoculation in **mice not given** cocoa drink. ^{dh}comparison between mean percentage parasitized RBCs in blood samples taken from **mice not given** cocoa drink on days two and six. ^{eh}comparison between mean percentage parasitized RBCs in blood samples taken from **mice not given** cocoa drink on days four and six.

ingested natural cocoa exerted anti-inflammatory and antioxidative properties that blunted the damage caused to hepatic sinusoids by ROS, oxidative stress, and immune-responsive proinflammatory factors engendered by *P. berghei* infection.

In the present investigation, challenging mice with *P. berghei*-parasitized RBCs resulted in a significant increase of serum ALT and AST levels, as compared with the control mice sera. The increased activities of the enzymes AST and ALT in serum indicate hepatocellular damage since the levels of these enzymes are elevated in acute hepatotoxicity.⁴⁰ Our findings that ALT and AST levels were lower in infected mice given cocoa compared with the infected mice not given cocoa, affirms implied hepatoprotective activity of components in cocoa. Other researchers such as Ko et al⁴¹ have shown that antioxidants reduce AST and ALT levels in experimental rats and mice with liver disorders. Furthermore, extract of *Antrodia camphorata* (an antioxidant) has been reported to ameliorate the increase in ALT and AST levels caused by chronic repeated carbon-tetrachloride intoxication in mice, by mediating antioxidative and free-radical scavenging activities.⁴² Our finding that serum AST and ALT could not distinguish between infected mice given cocoa and

control mice, provokes an inference that these functional markers are less acute indicators of hepatic damage than MHSd, and/or may lag behind structural damage to the liver.

There is a possibility that apart from polyphenols, other components in cocoa such as methylxanthines, peptides and minerals,⁴³ may have accounted individually or synergistically for the observed hepatoprotective activity of ingested cocoa in the present study. However, support for antioxidative mediation of the observed liver protection in the mice that drank cocoa in the work being reported, resides in the well-established role of oxidative stress in the pathophysiological processes of liver injury.^{44,45} Despite recent skepticism, literature abounds with the in vivo antioxidative benefits of the polyphenols in cocoa.^{43,46,47} Moreover, our own laboratory has a previous study which provides supportive evidence for in vivo antioxidative benefit of ingested cocoa.⁴⁸ In that study streptozotocin-induced diabetic rats were given 2% Goodfood natural cocoa powder for 13 weeks. The concentration of plasma isoprostane, an oxidative marker, rose by 43.82% in streptozotocin-induced diabetic rats and by only 9.14% in streptozotocin-induced diabetic rats that voluntarily ingested cocoa as a drink,

Table 4 Two-way ANOVA comparisons of percentage of parasitized RBCs coincident with or without cocoa ingestion in mice infected with *Plasmodium berghei*

Source of variation	Percentage of total variation	Probability (P) value	Degrees of freedom	Sum of squares	F value
Interaction effects	1.61	0.3138	2	209.0	1.185
Access to cocoa drink	1.16	0.1967	1	151.4	1.710
Number of days post inoculation	60.80	<0.0001	2	7,926.0	44.76
Residual (error)	–	–	52	4604	–
Total			57		

Notes: Sample sizes and descriptive statistics given in Table 2.

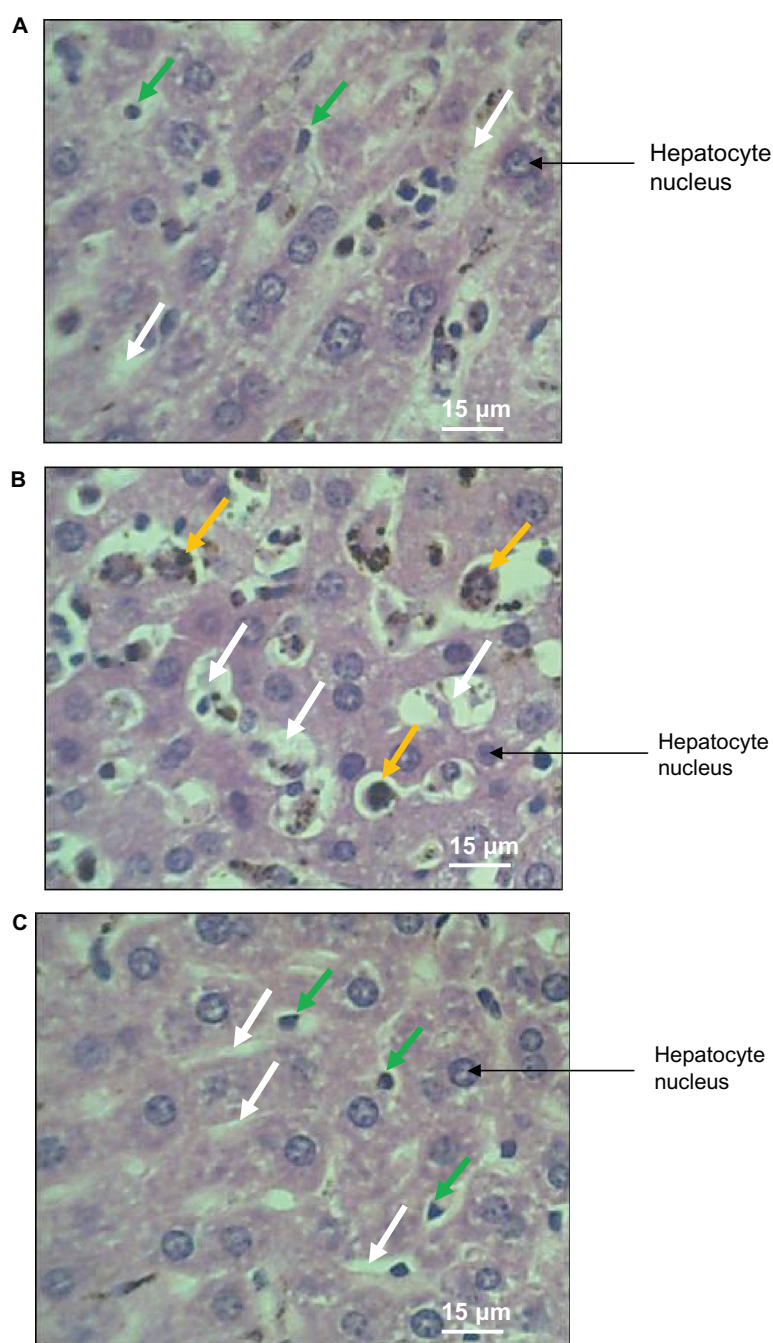
Abbreviations: ANOVA, analysis of variance; RBC, red blood cell.

Table 5 Bonferroni post hoc comparisons of percentage of parasitized RBCs coincident with or without cocoa ingestion in mice infected with *Plasmodium berghei*

Number of days after inoculation of mice with parasitized RBCs	Mean percentage parasitized RBCs		Difference	T-value	Probability (P) value	95% confidence interval of the difference
	Mice given access to cocoa drink	Mice not given access to cocoa drink				
2	1.726	5.190	3.464	0.8231	$P > 0.05$ (ns)	-16.947-13.87
4	13.45	11.86	1.590	0.3658	$P > 0.05$ (ns)	-12.28-9.115
6	27.63	35.45	7.820	1.810	$P > 0.05$ (ns)	-2.872-18.52

Note: Sample sizes are presented in Table 2.

Abbreviations: RBC, red blood cell; ns, not significant.

**Figure 2** H and E stained sections showing liver architecture and sinusoids of (A) infected mouse given cocoa, (B) infected mouse not given cocoa, and (C) control mouse. White arrows indicate hepatic sinusoids. Kupffer cells (orange arrows) in (B) have undergone hypertrophy compared with those in (A) and (C) green arrows.

signifying a 34.68% decrease in the plasma oxidative marker. Furthermore, Etsey⁴⁸ showed that total antioxidative power of testicular tissue, measured as a uric acid equivalent, decreased by 80.5% in animals not given cocoa versus 43.4% in animals that drank cocoa over 13 weeks of the study. This indicated that ingestion of 2% natural cocoa powder coincided with 37.1% retention of total antioxidative power in the testicular tissue of streptozotocin-induced diabetic rats.

It is noteworthy that cocoa intake failed to ameliorate progression of parasitemia in the present study. On the one hand, it indicates that the hepatoprotective benefit observed did not result from less severe parasitemia in the mice that drank cocoa. On the other hand, it provokes the inference that cocoa intake did not treat the malaria infection in the mice. Questions arise as to whether this finding resulted from failure of the mice to ingest an adequate amount of cocoa, or whether the unnatural concentration of parasites in the inoculum exceeded the antiparasmodial activity of ingested cocoa. Future studies would seek to address these issues. Although postprandial plasma concentrations of cocoa metabolites were not determined in our study, it has been reported that recovery of total polyphenol was 100% in the prepared hot cocoa drink.⁴⁹ It is therefore unlikely that the mode of preparation of cocoa in our study reduced the quantity of available polyphenols in the drink ingested by the mice. Regardless of the absence of antimalarial activity in the present report, it unequivocally demonstrated that natural cocoa ingestion has potential for controlling liver damage coincident with malaria parasitemia. Pertinently, our laboratory has reported a comparable finding that voluntary ingestion of natural cocoa extenuated hepatic injury in rats with experimentally induced chronic alcoholic toxicity.⁵⁰

Conclusion

Voluntary intake of 2% aqueous suspension of natural cocoa powder significantly reduced structural and functional damage of the liver in mice infected with *P. berghei* for six days. Given the known role of oxidative stress and inflammation in liver injury in general and referable to plasmodium parasitemia in particular, it is postulated that the hepatoprotective activity of natural cocoa is attributable to its antioxidative/anti-inflammatory properties. Available literature suggests that theobromine and polyphenols in cocoa may have exerted antioxidative/anti-inflammatory activity that blunted pernicious activity of ROS and proinflammatory immune-response macromolecules generated in infected mice. This study does not rule out the possibility that other components in natural cocoa may have produced, or contributed to, the hepatoprotective benefit reported via nonantioxidative properties.

Acknowledgments/disclosure

FK Addai is nationally (Ghana) and regionally (West Africa) known as a researcher and promoter of natural cocoa consumption for better health. He is the Founder and Director of Kakawa Enterprise Limited, an ultramicroscale company that produces GoodFood Unsweetened Natural Cocoa Powder. Kakawa Enterprise Limited made no financial contribution towards the study or publication of this paper.

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