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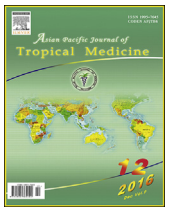

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Asiatic acid-pectin hydrogel matrix patch transdermal delivery system influences parasitaemia suppression and inflammation reduction in *P. berghei* murine malaria infected Sprague–Dawley rats

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ABSTRACT

Objective: To report the influence of transdermal delivery of asiatic acid (AA) in *Plasmodium berghei*-infected Sprague Dawley rats on physicochemical changes, %parasitaemia and associated pathophysiology.

Methods: A topical once-off AA (5, 10, and 20 mg/kg)- or chloroquine (CHQ)-pectin patch was applied on the shaven dorsal neck region of *Plasmodium berghei*-infected Sprague Dawley rats (90–120 g) on day 7 after infection. Eating and drinking habits, weight changes, malaria effects and %parasitaemia were compared among animal groups over 21 d.

Results: AA-pectin patch application preserved food and water intake together with % weight gain. All animals developed stable parasitaemia (15–20%) by day 7. AA doses suppressed parasitaemia significantly. AA 5 mg/kg patch was most effective. AA and CHQ displayed bimodal time-spaced peaks. CHQ patch had a longer time course to clear parasitaemia.

Conclusions: AA influences bio-physicochemical changes and parasitaemia suppression in dose dependent manner. In comparison by dose administered, AA has much better efficacy than CHQ. AA may be a useful antimalarial. AA and CHQ displays bimodal peaks suggesting possible synergism if used in combination therapy.

1. Introduction

Malaria still "rules the roost" in terms of the number of deaths it causes in children under five years and pregnant women, literally killing the unborn child [1,2]. The morbidities and mortality are caused by the parasite but much more by the pathophysiology of malaria or inadequate treatment, drug resistance, and drug induced toxicities [3]. Convoluted treatment regimens of malaria and frequency of dosing contribute significantly to malaria management failure [4]. Oral drug delivery is the major route by which most medication (74%) is administered as well as the standard method by which efficacy of a therapeutic agent is determined although the efficiency of this system is inadequate [5]. Novel methods of drug delivery, therefore become necessary to improve drug bioavailability and therapeutic efficacy [6]. Transdermal drug delivery system (TDDS) (the patch) has emerged as one of the novel means by which antimalarial drug may be delivered through the skin for systemic effects after overcoming the morphological, biophysical, and physicochemical characteristic of the skin barrier [7]. Advantages of TDDS include non-invasiveness, reduced dosing frequency, reduction of first pass hepatic metabolism, therapeutic enhancement and maintenance of steady state of drug concentrations in plasma [8–10]. Studies are ongoing on optimization of TDDS which has been patented for insulin and chloroquine (CHQ) delivery in diabetes mellitus and malaria, respectively [11,12]. The use of TDDS in malaria treatment with CHQ in a murine malaria model of *Plasmodium berghei* (*P. berghei*) was suggested to improve efficacy and ameliorate

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malaria pathophysiology [13]. Experimentation with maslinic acid (MA) and oleanolic acid (OA) TDDS has been embarked on (unpublished data). Furthermore, an oral aqueous-organic suspension of asiatic acid (AA, 10 mg/kg) has recently been shown to retard parasitaemia proliferation and a failure for %parasitaemia reach patent levels in a 21 d sub-chronic study [14] although the mode of action was not explored. AA is a triterpene with known anti-inflammatory and antinociceptive [15], anti-hyperglycaemia [16], immunoregulatory [17], haemodynamic modulation [18], inhibition of aberrant cell proliferation [19], anti-oxidant and pro-oxidant [20,21], and lipid peroxidation ameliorative properties [22].

The general principle of TDDS which makes it attractive in malaria treatment is the avoidance of single-pass hepatic metabolism and possible degradation that allows drugs to be delivered into the circulation directly, the smaller doses used and possible increased drug bioavailability in plasma. Buoyed by this knowledge, formulation (with some modifications) of an AA-pectin hydrogel matrix patch for the delivery of the amphiphilic triterpene via the skin was envisaged as an improved method for increased efficacy of the triterpene shown to be an effective suppressor of parasitaemia. The TDDS's sustained and controlled release of curative molecules was hypothesized as possible with AA due to its relatively moderate solubility in aqueous environment and its amphiphilic nature. Consequently, in a bid to increase AA bioavailability, reduce dosing frequency with concurrent diminution of the delivered dose, we purposed to investigate the effects of TDDS delivered AA in *P. berghei*-infected young (90–120 mg/kg) male Sprague Dawley (SD) rats. Here we present our findings on the influence of AA-pectin hydrogel matrix patch on food and water intake as well as %weight gain, its effects on malaria pathophysiology and parasitaemia reduction in a sub-chronic (21 d) study.

2. Materials and methods

2.1. Materials

2.1.1. Drugs, chemical and accessories

Different sources and suppliers were used for the procurement of drugs, chemicals and accessories but AA (>97% purity), Giemsa stain, dimethyl sulphoxide (DMSO), CHQ diphosphate were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals and reagents were of analytical grade.

2.1.2. Animals

Male SD rats (90–120 g) were obtained from Biomedical Research Unit (BRU) at University of KwaZulu-Natal where they were bred and housed for the entire experiment period. The animals were kept under maintained laboratory conditions of constant temperature [(22 ± 1) °C]; CO_2 (<5 000 ppm), humidity of (55 ± 5)% and illumination (12 h light/dark cycles). The animals had full access to food, standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and water *ad libitum*. Animals were sacrificed by isofor (100 mg/kg) inhalation anaesthesia (Safeline Pharmaceuticals, Rooderport, South Africa) in a gas chamber by day 12 (infected none treated) and by day 21 (non-infected and infected treated animals). All experiments and protocols used in this study were reviewed and approved by the animal ethics committee of the University of KwaZulu-Natal (UKZN) with ethical clearance numbers 079/14/Animal and 013/15/Animal issued.

2.1.3. Plasmodium murine malaria model

CHQ-susceptible strain of *P. berghei* ANKA, murine malaria parasite was a kind donation from Professor Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). The parasite was sub-cultured, harvested, and stored in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at −80 °C until use.

2.1.4. Experimental protocol design

Animals were inoculated at day 0, treated at day 7 and monitored up to day 12 for infected but non-treated control group (IC group) and day 21 for the non-infected control group (NIC group) and AA groups including AA-pectin patch applied groups at 5, 10, and 20 mg/kg doses. Animals groups sacrificed on day 0, 3, 9, 12, and 21. The protocol design was shown in Figure 1.

2.2. Methods

2.2.1. Induction of parasitaemia

P. berghei [10^5 parasitized red blood cells (pRBC's) suspension in saline] was used as an intraperitoneal (ip) inoculum [23]. Control animals received equivalent amount of saline.

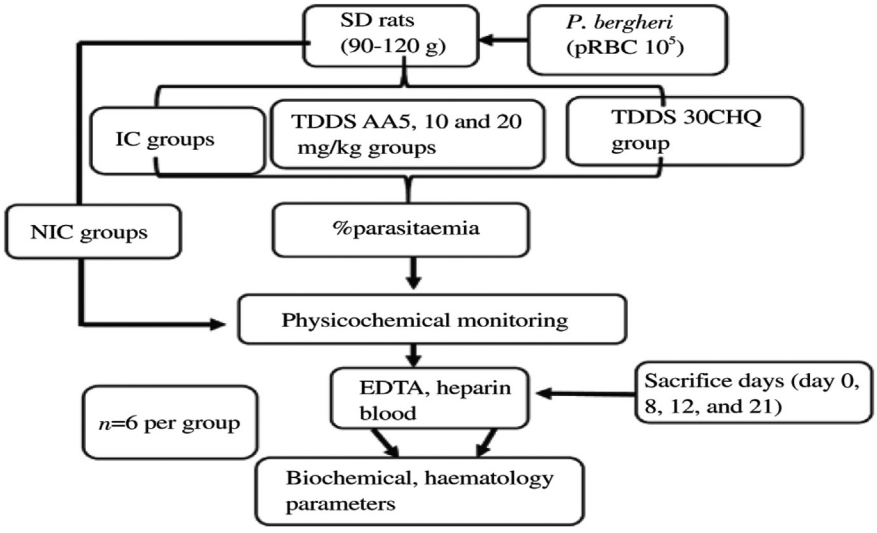

Figure 1. Flow diagram for the experimental protocol and design.
30CHQ was the 30 mg/kg CHQ TDDS treated group.

2.2.2. Parasitaemia monitoring

Peripheral blood was obtained by tail prick method and thin smears of rat blood were used in monitoring parasitaemia. Malaria monitoring consisted of Giemsa staining of the thin smear and examination under a microscope (Olympus Cooperation, Tokyo, Japan). The actual number of pRBC's relative to 2×10^4 RBC's was used to calculate %parasitaemia [24]. Change in % parasitaemia was monitored at 72 h (pre-patent period), every third day up to day 7 (patent period) [25], every day during treatment period for five days and thereafter every other day post-treatment period until day 21.

2.2.3. Evaluation of parasitaemia

Appearance of parasites in the blood after ip inoculation takes 2–3 d [24]. Two set points were used in the study to determine the performance of the malaria model. These were time-point at which malaria parasites were first detected in peripheral blood (pre-patent) which was 72 h post-infection and a parasitaemia of 15–20% on day 7, without intervention [26], signifying a stable state of severe malaria (SM).

2.2.4. Preparation of pectin patches for transdermal delivery system

Amidated pectin hydrogel matrix patches were prepared using a previously described protocol by Musabayane *et al.* [7], with slight modifications. Low methoxyl amidated pectin with a degree of esterification (DE) of 23% and an amidation of 24% was used for the preparation of the AA and CHQ patches. In separate beakers, water (110 mL) was used to dissolve either 30 mg/kg CHQ or AA (5, 10, and 20 mg/kg) with pectin (4.4 g) and agitated at 37 °C in a water bath at a speed of 38× G using an electric motor mixing rotor (Heidolph instruments GmbH & Co. KG, Schwabach, Germany) for 15 min. DMSO, vitamin E, and eucalyptus oil were added to the mixture, sequentially with continuous mixing. The mixture (11 mL) was pipetted into petri dishes and frozen at −81 °C for 18 h, following which a 2% $CaCl_2$ solution was added at room temperature (±25 °C) for matrix cross-linking. The patches were stored at 4 °C until use.

2.2.5. Application of the patch

Three discs [(4 ± 1) mm^2] were punched out from the different AA concentration (5, 10, and 20 mg/kg) patches or CHQ (30 mg/kg) and applied once-off onto the shaved dorsal region of the animal from day 7 to day 10 (three days). The jacket holding the patch in place was made from clinically sterile adhesive fabric plaster (Mediplast, Neomedic, Rickmansworth, and Herefordshire, UK) which caused no discomfort to the animals. The dorsal neck region was selected because it was the least accessible to the animal grooming habits and avoided removal of the patch by the animal. The TDDS aimed at reducing phytochemical amount delivered to the animal, dosing frequency, treatment duration and general animal discomfiture. A theoretical total AA yield of patch was estimated at 1 μg/per disc for 5 mg/kg AA dose and 3 μg was administered, therefore, per animal once-off to provide five day treatment. Other doses were multiples of this calculation.

2.2.6. Influence of AA-pectin patch application on % parasitaemia

A comparison was made between the different doses of topical AA (5, 10, and 20 mg/kg)-pectin patch, 30CHQ-pectin patch and the IC on changes of %parasitaemia over the 21 d period.

2.2.7. Haematological analysis

All animals were sacrificed by exposing to isofor (100 mg/kg) inhalation anaesthesia (Safeline Pharmaceuticals, Roodeport, South Africa) on day 0, 9, 12 and at the end of the study at day 21. Blood samples were collected by cardiac puncture into Na_2EDTA tubes for whole blood analysis. Liver, kidney, muscle and heart were removed, snap frozen in liquid nitrogen and stored together with the plasma in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at −80 °C until use in other studies.

2.2.8. Influence of AA-pectin patch on inflammation

Whole blood was analysed in a haematology four part white cell count (WBC) differential analyser (Coulter-Bachmann). WBC levels were used to estimate inflammatory response to malaria in AA-pectin patch applied animals, the NIC, IC, and CHQ-patch treated groups over time (21 d).

2.2.9. Influence of AA on inflammation in malaria C-reactive protein (CRP)

Inflammation is the bed rock to malaria pathophysiology. To indicate the influence AA (10 mg/kg) on inflammation, CRP was estimated using the Elabscience Rat hs-CRP (High Sensitive C-Reactive Protein) ELISA kit Catlog No: E-EL-R0506 (Elabscience Biotechnology Co. Ltd ELISA, Wuhan, P.R.C.) according to the manufacturer's instruction.

2.2.10. Influence of AA on severe malaria anaemia (SMA)

Anaemia presents as a low red blood cell mass relative to plasma. Haematocrit (Hct) is a compartmentalization of cellular components of whole blood expressed as a percentage such that the lower the percentage, the more severe the anaemia. Hct, therefore, as a surrogate marker for SMA was compared amongst the groups of topical AA (5, 10, and 20 mg/kg)-pectin hydrogel matrix patch applied, 30CHQ-pectin matrix patch treated, the NIC and the IC groups.

2.3. Statistical analysis

Unless otherwise stated, data was presented as standard error of the mean (SEM). Statistical comparisons performed by one way analysis of variances (ANOVA), followed by Tukey–Kramer multiple comparison ad hoc test using Graph-pad InStat Software (version 5, GraphPad Software, San Diego, California USA). A $P < 0.05$ was considered statistically significant difference.

3. Results

3.1. Influence of AA on bio-physicochemical properties

Results of animals applied a once-off topical AA (5, 10, and 20 mg/kg)-pectin hydrogel matrix patch on changes of eating, drinking habits and %weight gain during the 21 d sub-chronic study were shown in Figure 2A, B and C. AA-pectin patch significantly preserved food and water intake together with increased %weight gain compared to IC and 30CHQ patch

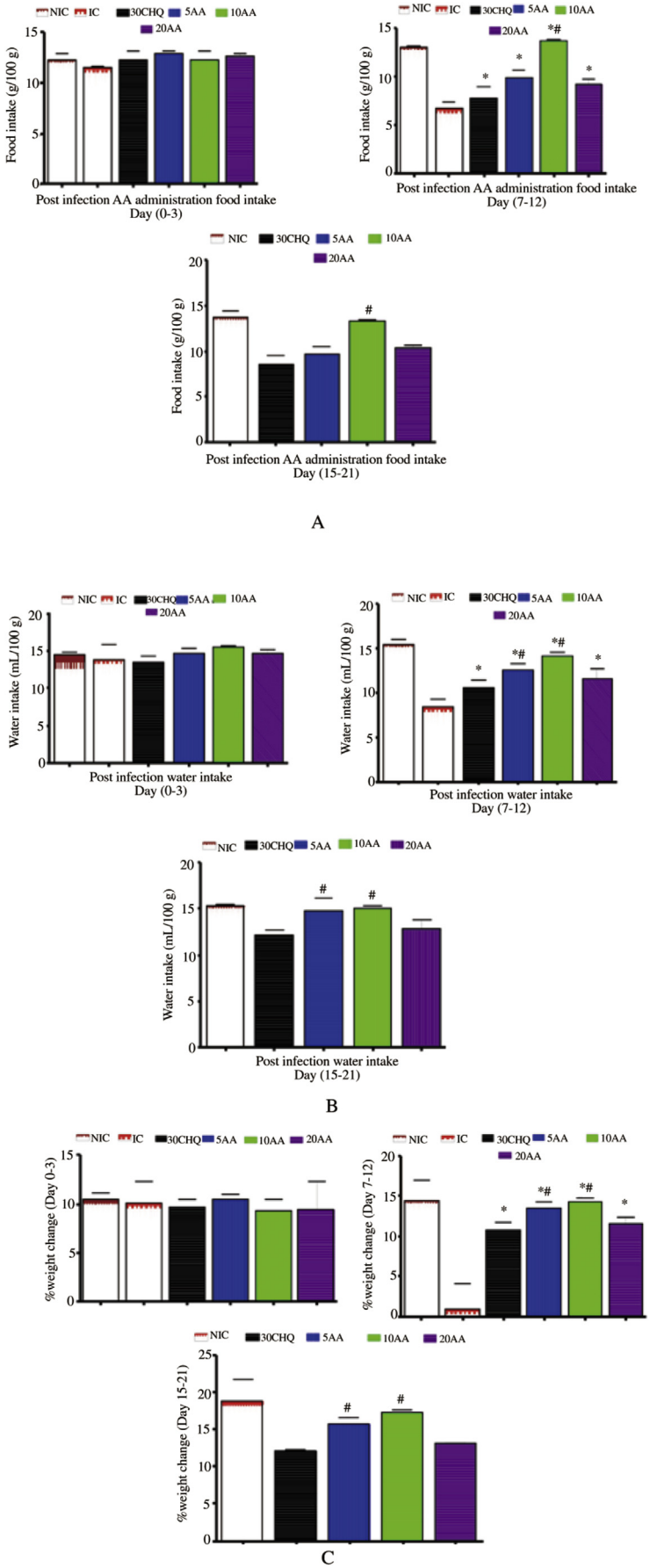

Figure 2. Comparison of AA influence on food intake, water take and %weight change over 21 d period (n = 6).
IC was infected but non-treated control group; NIC was non-infected control group; 30CHQ was the 30 mg/kg CHQ TDDS treated groups; 5AA, 10AA, and 20AA represented AA-pectin patch applied groups at 5, 10, and 20 mg/kg doses, respectively. A: food take; B: water take; C: %weight. $^{*}P < 0.05$, compared to IC; $^{\#}P < 0.05$, compared to 30CHQ patch.

($P < 0.05$, respectively) during the treatment period. Compared to 30CHQ patch, IC significantly decreased food and water intake as well as %weight gain ($P < 0.05$) by day 12. Compared to the NIC, 30CHQ patch had significantly decreased food and water intake as well as %weight gain ($P < 0.05$) by day 21.

3.2. Validation of parasitaemia

Comparison of the various time points against the animal groups' %parasitaemia was shown in Table 1. AA (5, 10, and 20 mg/kg)-pectin patch application had significantly higher % parasitaemia at day 7 compared to the 30CHQ-pectin patch treatment ($P < 0.05$). Compared to the IC, AA (5, 10, and 20 mg/kg)-pectin patch had significantly lower %parasitaemia at peak period ($P < 0.05$). Time to peak %parasitaemia was significantly longer in 5AA, 10AA, and 20AA-pectin patch application than 30CHQ-pectin patch treatment ($P < 0.05$). AA (5 mg/kg)-pectin patch had a significantly lower peak %parasitaemia compared to 30CHQ-pectin patch ($P < 0.05$). Compared to the IC, 30CHQ patch had a significantly lower peak %parasitaemia ($P < 0.05$). Parasitaemia suppression continued until parasites were non-detectable by microscopy at day 18 for 5AA administered animals but persisted in 30CHQ treated animals.

3.3. AA-pectin patch application influence on % parasitaemia

A comparison of the influence of AA-pectin patch with controls was given in Figure 3. AA (5, 10, and 20 mg/kg)-pectin patch significantly reduced parasitaemia compared to IC ($P < 0.05$) at all relevant time points. In comparison to the 30CHQ-pectin patch treatment, AA (5 mg/kg)-pectin patch significantly lowered parasitaemia ($P < 0.05$) at all pertinent time points. Compared IC, 30CHQ patch had significantly lower %parasitaemia ($P < 0.05$) at day 10 and 12. AA and CHQ administration displayed significantly different bimodal peak parasitaemia time points.

3.4. AA-pectin patch application and %parasitaemia-time area under the curve (AUC)

Area under the curve for %parasitaemia and time [$AUC_{(0–21\ d)}$] showed the time course of AA administration relative to controls. A comparison of the time-course influence of AA on malaria was made in Figure 4. AA (5 mg/kg)-pectin patch displayed significantly lower $AUC_{(0–21\ d)}$ compared to the $ICAUC_{(0–12\ d)}$ ($P < 0.05$). Compared to the 30CHQ, AA (5, 10 mg/kg)-pectin patch had significantly lower AUC($P < 0.05$). Despite a shorter time period, $ICAUC_{(0–12\ d)}$ was equal to 10AA $AUC_{(0–21\ d)}$.

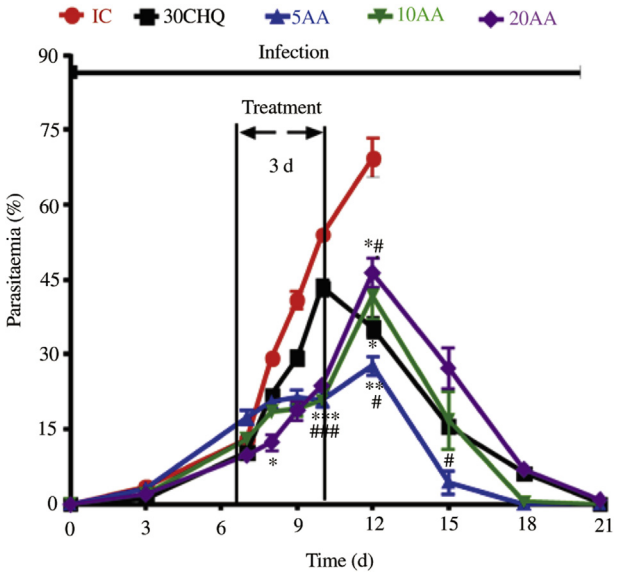

Figure 3. TDDS AA influence on %parasitaemia compared to controls ($n = 6$).
*$P < 0.05$, compared to the IC; #$P < 0.05$, compared to CHQ control.

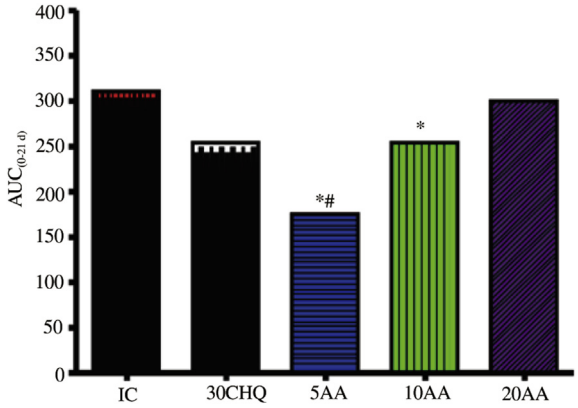

Figure 4. Area under the %parasitaemia-time curve [$AUC_{(0–21\ d)}$] for % parasitaemia in the pre-infection AA transdermal delivery system AA administration protocol ($n = 6$).
*$P < 0.05$, compared to the IC; #$P < 0.05$, compared 30CHQ control.

3.5. AA-pectin patch influence on inflammation

WBC count showed the presence or absence of immune response and/or inflammation. The effect of AA-pectin patch application on WBC counts over the 21 d study was compared to control in Table 2. At relevant time points AA (5, 10 mg/kg)-pectin patch significantly reduced WBC count compared to IC ($P < 0.05$). AA (5 mg/kg)-pectin patch reduced WBC count significantly in comparison to 30CHQ ($P < 0.05$) at all relevant time points. AA (10, 20 mg/kg)-pectin patch decreased WBC

Table 1
Comparison of %parasitaemia at different time points among animal groups ($n = 6$).

Protocol	Dose	Pre-patent parasitaemia (h)	%parasitaemia day 3	%parasitaemia day 7	Parasitaemia at peak (%)	Peak period (d)	Parasitaemia suppression (d)
Post-infection TDDS administration	IC	72	3.5 ± 0.6	13.0 ± 3.3	73.3 ± 6.7	N/A	N/A
	30CHQ	72	2.3 ± 0.3	10.3 ± 1.1	45.7 ± 1.8*	9	21>
	5AA	72	3.9 ± 2.2	21.3 ± 3.7#	24.5 ± 2.6*#	12#	<18
	10AA	72	1.9 ± 0.8	18.4 ± 3.7#	43.6 ± 4.5*	12#	18
	20AA	72	2.6 ± 2.4	12.4 ± 4.6	47.5 ± 3.4*	12#	18>

*$P < 0.05$, compared to IC; #$P < 0.05$, compared to 30CHQ. N/A means this criteria is not applicable; 18 means suppression of parasitaemia was attained on day 18; <18 and 18> means parasitaemia suppression was obtained before and after day 18, respectively; 21> means parasitaemia suppression was not attained at day 21.

Table 2
Comparison ofWBC count at pre-patent, patent, treatment, and post-treatment periods amongst the different animal groups (n = 6).

Protocol	Dose	Pre-patent period WBC	Patent period WBC	Treatment period WBC	Post-treatment period WBC
Post-infection TDDS treatment	NIC	4.6 ± 0.3	4.4 ± 0.2	4.4 ± 0.2	4.4 ± 0.2
	IC	4.5 ± 0.3	14.6 ± 0.5	48.2 ± 3.3	N/A
	30CHQ	4.8 ± 2.8	17.6 ± 3.5$^{\alpha}$	36.1 ± 4.9$^{\alpha}$	10.8 ± 1.9$^{\alpha}$
	5AA	4.7 ± 0.8	7.1 ± 1.9$^{*\#}$	6.8 ± 0.1$^{*\#}$	4.5 ± 0.3$^{\#}$
	10AA	4.7 ± 0.1	10.7 ± 1.6$^{*\#}$	25.8 ± 2.5$^{*\#}$	3.4 ± 3.1$^{\#}$
	20AA	4.3 ± 0.1	14.9 ± 3.2$^{\alpha}$	34.9 ± 4.3$^{*\#}$	2.5 ± 0.2$^{\#\alpha}$

$^{\alpha}P < 0.05$, compared to NIC; $^{*}P < 0.05$, compared to IC; $^{\#}P < 0.05$ compared to 30CHQ.

count significantly compared to 30CHQ ($P < 0.05$). Compared to the IC, 30CHQ patch had significantly higher WBC count ($P < 0.05$) at all relevant times.

3.6. Plasma CRP

CRP depicted the presence or absence of inflammatory response in malaria. The influence of 10AA on CRP concentration compared to controls in Figure 5. The 10AA administration reduced CRP concentrations significantly compared to the IC and CHQ treated animals ($P < 0.05$). There was no relative change in NIC compared to either the NIC or the CHQ.

3.7. AA-pectin patch influence on SMA

Haemtocrit was a positive indicator of anaemia presence. The influence of AA (5 mg/kg)-pectin patch on Hct over the duration of the study was made in Figure 6. Compared to the IC, AA (5 mg/kg)-pectin patch significantly preserved Hct ($P < 0.05$) during treatment and post-treatment periods. AA (5 mg/kg) preserved Hct compared to 30CHQ ($P < 0.05$) at all relevant time points. The 30CHQ patch had significantly lower Hct compared to the NIC ($P < 0.05$) (Figure 6).

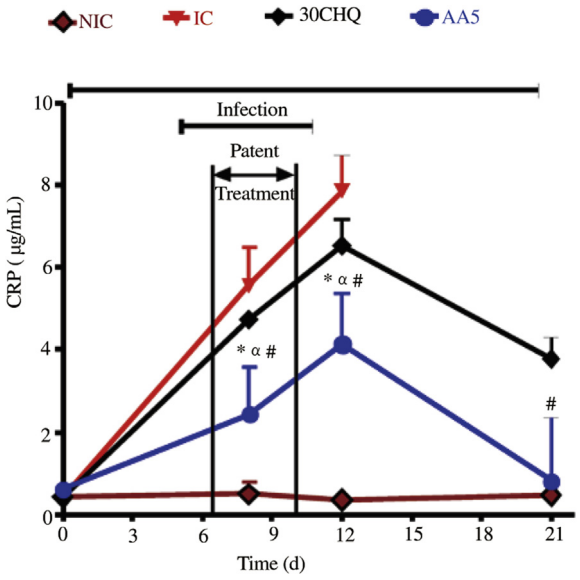

Figure 5. Changes of CRP concentration in 10AA administered animals compared to controls.
αP < 0.05, compared to the NIC; $^{*}P < 0.05$, compared to IC; $^{\#}P < 0.05$, compared to CHQ.

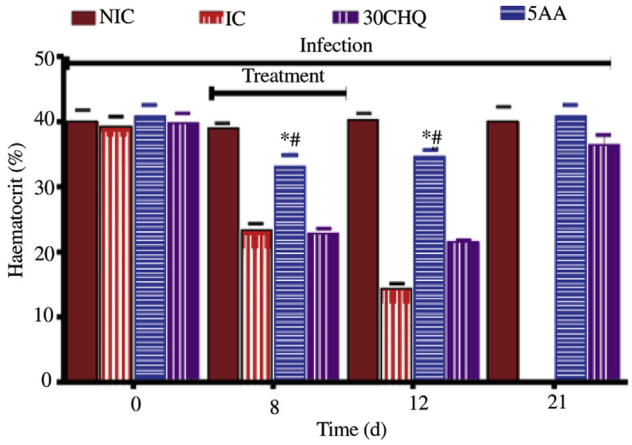

Figure 6. Comparison of AA-pectin patch application influence on Hct amongst the different animal groups (n = 6).
$^{*}P < 0.05$, compared to IC; $^{\#}P < 0.05$, compared to 30CHQ.

4. Discussion

Natural AA has relatively moderate dissolution factor in aqueous media that subsequently results in low bioavailability when administered by the oral route [27]. Pursuant to the quest of increasing the bioavailability of AA, a novel method of delivering the phytochemical in *P. berghei*-infected SD male rats was developed in this study.

Using young, 6 weeks old SD rats (90–120 g) which displayed SM, SMA, and in some CM [26,28] topical application of AA (5, 10, 20 mg/kg)-pectin hydrogel matrix patch suppressed parasitaemia to undetectable levels by day 21 post-infection and preserved the biophysical properties of the animals in the face of SM infection. Preservation of eating and drinking habits by TDDS AA administration may have had profound effect on the pathophysiology in general. This phenomenon is corroborated by findings that pre-infection AA 10 mg/kg oral administration retarded patent parasitaemia development with *P. berghei* infection [14]. In a similar report, post-infection AA (10 mg/kg) influenced glucose metabolism favourably [29]. The %parasitaemia showed bimodal peaks with 30CHQ and AA-pectin patches showing peaks at day 10 and 12, respectively. AA5-pectin patch displayed a significantly lower %parasitaemia-time area under the curve [$AUC_{(0-21\ d)}$]. Of note is that even though the IC animals were sacrificed by day 12 the % parasitaemia-time course was markedly higher to that of 5AA and 10AA $AUC_{(0-21\ d)}$, but equal to 20AA $AUC_{(0-21\ d)}$ showing that the administration of AA may have influenced this phenomenon. Indeed, this was also apparent with CHQ treatment

which posted comparable $AUC_{(0–21\ d)}$ to 10AA which was significantly higher that the 5AA $AUC_{(0–21\ d)}$. Compared to the 30CHQ patch, the drug load and efficacy ratio was favourable towards 5AA, equal to 10AA, and lower for 20AA when the phytochemical was administered by TDDS. The disparity observed needs further clarity through more investigations with special emphasis on the molecular dynamics and interactions of key proteins in the malaria parasite to AA.

Infection effectuation by an ip inoculation of 10^5 pRBC's saline suspension resulted in SM indicated by decreased Hct in the IC. There was a clear distinction of the influence of AA-pectin patch on SM when comparing to changes from day 7–21. In malaria, anaemia changes precede the actual overt malaria with low blood count indices and increased plasma volume from increased fibrinogen synthesis by the liver to counterbalance low whole blood viscosity by increasing plasma viscosity resulting in little to insignificant change of whole blood viscosity in SMA with characteristic hypovolaemia [30]. This is in agreement with observations that the rapture of pRBC's upon merozoite release play minor role in the early induction of anaemia which is contributed mainly by the loss of npRBC's [31] and impaired erythropoiesis [32]. Dyserythropoiesis on its own, however, may likely contribute little in SM aetiology [33] but will be a critical and predominant feature in chronic carriage of the parasite [34] where the haemodynamics studies revealed an initial hypovolaemia followed by hypervolaemia and normovolaemia [30]. Parasitaemia clearance does not automatically normalise Hct which may continue to fall [35] suggesting that other factors may be at play in the orchestration of malaria anaemia with increased destruction of RBC's and decreased synthesis. The production of hemozoin, a haemoglobin biocrystallization product, has also been implicated in immunological responses and bone marrow dyserythropoiesis [36] and cellularity manipulation towards myeloid lineage cell proliferation resulting in the erythroid lineage cell hypoplasia with consequent reduction in some haematological indices [37,38]. The observed Hct reduction, followed by subsequent resolution of SMA through AA intervention, is usually a chronic malaria processes in human beings.

Stretching the imagination somewhat, the 7th day when patent malaria was observed may actually be a chronic infection of 186.9 d [39], if the rat age to human conversion theorem is anything to go by [40]. Therefore, the anaemia displayed by Hct may have been a reflection of chronic anaemia of malaria which was controlled by the application of the AA-pectin patch. Furthermore, the reduced food intake, which may have been contributed by malaria anorexia and bitterness of CHQ [41], could have caused the reduction in Hct in IC and CHQ-pectin patch treated animals, respectively. While the TDDS of CHQ could have bypassed the gut where the bitter substance-sensing receptors lie, the entero-hepatic circulation may bring the drug into contact with the receptors several times over, with possibly the same effect. Consequently, animals treated with CHQ may tend to have appetite suppression which may be worsened by reduced drug inficacies. This understanding may counteracts earlier assertions that TDDS CHQ delivery camouflages the bitterness of the drug that could reduce compliance with treatment [7]. This may highlight the need to punctuate treatment with parenteral feeding when TDDS CHQ is used in malaria taking into consideration as well the hypoglycaemic effect of CHQ [42,43].

Administration of AA-pectin patch showed that the lower dose of AA (5 mg/kg) reduced %parasitaemia as well as inflammation and reduced leucocytosis to a significantly greater extent than the other two doses and 30CHQ. While there are possibly many reasons for this phenomenon, two speculative explanations are in order. First, the loading dose of AA in the pectin, although lower, may allow for a faster release of the phytochemical, accumulating relatively earlier in plasma than higher doses. However, diffusion kinetics will rather instruct that higher solute concentration have a higher capacity of delivery than the lower ones. Second, the higher doses carrying patches may result in the delivery of higher AA concentration to the extent that the anti-oxidant and pro-oxidant capacity of AA may favour parasite proliferation than its eradication. On the other hand lower patch with a lower dose may just deliver an optimum amount of AA to overwhelm parasite defences. Indeed, inflammatory processes in malaria are pivotal in the marshalling malaria pathogenesis. Inflammatory cytokines [macrophage migration inhibitory factor (MIF), tumour necrosis factor-α (TNF-α), IFN-γ, IL-12 may contribute to protection or pathology depending on production site, production timing, produced levels together with the presence or absence of immunoregulatory, *e.g.* cytokine IL-10 [25,44]. Unregulated expression of iNOS may result in vascular permeability, aberrant reactive oxygen species (ROS) production, peroxynitrite ($ONOO^-$) synthesis, sodium wasting. These lead to hypovolaemia and generalised tissue damage with multi organ failure, which are common in malaria. Stress is a common phenomenon in malaria which upregulates cortisol synthesis driven most likely by βHSD-1 with increases in gluconeogenesis and increase in glucose concentration [45] that increases oxidative stress (OS). Anorexia-induced cachexia invariably increases lipid breakdown and hyperlipidaemia upregulating HMG CoA reductase. Taken together, the anti-inflammatory, immunomodulatory, anti-oxidant, anti-hyperlipidaemic, and pro-oxidant properties if AA may have formulated the combined mechanism by which the phytochemical suppressed parasitaemia and ameliorated malaria pathophysiology.

The concept of inflammation in malaria was well articulated by the influence of AA (5, 10, and 20 mg/kg)-pectin patch application where both %parasitaemia, WBC count, and CRP were proportionally and progressively linked at corresponding time points. A fall in %parasitaemia was associated with a concomitant fall in WBC count and indirectly showing a decrease in inflammatory response. This may be plausible if inflammation may be regarded as a result of parasite antigen presentation to the innate immune system with subsequent production of inflammatory mediators that drive the cycle of more parasite production leading to more inflammation. The concentration of CRP throughout sub-chronic study indicated acute inflammation at day 12 that coincided with peak %parasitaemia, declining critically showing abrogation of inflammation or inflammatory insult by 5AA-pectin patch. Therefore, intervention that either infringes upon either the parasite or the inflammatory cascade or both may invariable cause a decline of the other by mutual association. In like manner, AA-pectin patch may have influenced both or either as we have observed a decline in %parasitaemia that has a reciprocal dose dependent with the least dose being the most efficacious of the three doses. However, the question of which parameter is affected first and then influence the other is fundamental in unravelling the effect of AA in malaria, or whether these are parallel processes.

The anti-inflammatory effect of AA has been reported in animal models and cell lines [46]. The 5AA-pectin hydrogel patch in decreasing WBC count and CRP was either influenced by a decrease in parasitaemia through abrogation of inflammation or retraction of inflammation from the disease milieu could have led to a decrease in parasitaemia. Similar phenomenon with AA has been shown where it induced activated T cell apoptosis to control fulminant hepatitis [17].

The pharmacodynamics of AA5 may be that the patch slow release maintained plasma AA concentration exerting both oxidative and the anti-oxidant effects concurrently. Hypothetically, the ratio of the oxidant and anti-oxidant capacity may dictate which facet of AA predominates. At medium (10 mg/kg) and higher (20 mg/kg) doses, TDDS may deliver higher levels of AA which may be toxic and exacerbate disease. This may account for the lower dose (5 mg/kg) being more effective compared to the medium and higher doses. Furthermore, WBC count was lowered in a dose dependent manner with the 20AA-pectin patch displaying the least count corresponding to a higher %parasitaemia-time $AUC_{(0–21\ d)}$ and lower %parasitaemia post-treatment decline. Moreover, AA has been observed to induce cytotoxic apoptosis and cell arrest in human breast cancer through the activation of extracellular signalling kinase (ERK) and p38 mitogen-activated protein kinase (p38MAPK) [47], which may be the process by which lowed WBC count were observed with 20AA-pectin patch. However, the exact anti-disease mechanism of 5AA-pectin patch needs further research.

While the formation of ROS, nitric oxide (NO), and $ONOO^-$ may abate and reduce inflammatory response, host immune system necessary for phagocytosis of pRBC's, may be compromised through WBC depletion with lowered antimalarial efficacy. This may, however, not explain how the lower dose of AA (5 mg/kg) had a higher antimalarial efficacy in TDDS when the higher doses did not, contrary to our anticipation had lower efficacy. With oral AA administration it was observed in recent studies that the medium dose (10AA) was most efficacious in retardation of parasitaemia progression and control of malaria pathophysiology [14].

Bimodal peaks in %parasitaemia were conspicuous in this study. Treatment with 30CHQ patch had a steep rise in parasitaemia peaking at day 10 and gradually descending. On the other hand, AA-pectin patch displayed a reciprocal packing order with %parasitaemia precipitously decreasing in animals to which 5AA-pectin patch was applied. The shorter application time of three days for both AA and CHQ, compared to the five day treatment of twice daily dosing with CHQ and once daily with AA in po administration, portrayed a possible superior efficacy of TDDS in malaria. The bimodal peaks may show that the two compounds have different pharmacodynamics which could work well as double or triple combination therapy to avert drug resistance and prolong the useful life span of novel antimalarials [2].

In conclusion, our data demonstrates AA influence on % parasitaemia in murine malaria of SD rats. AA may have both anti-parasitic and anti-malarial disease activities supressing the parasite while ameliorating infection-induced pathology. Administration of AA post-infection suppressed parasitaemia by TDDS with the AA5-amidated pectin hydrogel matrix patch showing the most efficacy in anti-inflammatory response suppression, SMA amelioration, and general physicochemical properties outcomes. This may suggest that the 5AA-pectin patch may have both anti-parasitic and anti-disease facets necessary for a wholesome approach to malaria treatment seeing that no other supportive treatment was given to these animals besides patch application. The display of two distinct peak times in %parasitaemia progression may be attributable to different modes of action of the two compounds at the experiments concentrations and dosing regimens. Preservation of food and water intake, as well as increase in %weight gain in 5AA-pectin patch applied animals may indicate that the animals' appetite was not affected by sickness behaviour seen to be common in inflammatory and cachectic conditions like malaria.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Guyatt HL, Snow RW. Impact of malaria during pregnancy on low birth weight in sub-Saharan Africa. *Clin Microbiol Rev* 2004; **17**(4): 760-769.
[2] World Health Organization. *World malaria report 2013*. Geneva: WTO Press; 2013.
[3] National Institute of Allergy and Infectious Diseases. *Understanding malaria: fighting an ancient war*. New York: U.S. Department of Health and Human Services: National Institutes of Health; 2007.
[4] Burrows JN, van Huijsduijnen RH, Möhrle JJ, Oeuvray C, Wells TN. Designing the next generation of medicines for malaria control and eradication. *Malar J* 2013; **12**: 187.
[5] Shingade GM, Quazi A, Sabale PM, Grampurohit ND. Review on: recent trend on transdermal drug delivery. *J Drug Deliv Ther* 2012; **2**(1): 66-75.
[6] Kumar P, Philip A. Modified transdermal technologies: breaking the barriers of drug permeation via the skin. *Trop J Pharm Res* 2007; **6**(1): 633-644.
[7] Musabayane CT, Munjeri O, Matavire TP. Transdermal delivery of chloroquine by amidated pectin hydrogel matrix patch in the rat. *Ren Fail* 2003; **25**(4): 525-534.
[8] Wadhe K, Kalsait R, Umekar M. Alternate drug delivery system: recent advancement and future challenges. *Arch Pharm Sci Res* 2009; **1**: 297.
[9] Langer R. Drug delivery and targeting – the design of degradable materials and the development of intelligent delivery systems have had an enormous impact on drug-based therapies. *Nature* 1998; **392**(6679 Suppl): 5-10.
[10] Bharadwaj S, Garg VK, Sharma PK, Kumar N. Recent advancement in transdermal drug delivery system. *IJPPR* 2011; **2**(1): 247-254.
[11] Hadebe SI, Ngubane PS, Serumula MR, Musabayane CT. Transdermal delivery of insulin by amidated pectin hydrogel matrix patch in streptozotocin-induced diabetic rats: effects on some selected metabolic parameters. *PLoS One* 2014; **9**(7): e101461.
[12] Musabayane C, Van Heerden FR, Mukaratirwa S, Tufts MA. Transdermal delivery devices. US20150094259A1 (Patent) 2015.

[13] Murambiwa P, Tufts M, Mukaratirwa S, Van Heerden FR, Murabayane CT. Evaluation of efficacy of transdermal delivery of chloroquine on *Plasmodium berghei*-infected male Sprague–Dawley rats and effects on blood glucose and renal electrolyte handling. *Endocr Abstr* 2013; **31**: P203.

[14] Mavondo GA, Mkhwananzi BN, Mabandla MV. Pre-infection administration of asiatic acid retards parasitaemia induction in *P. berghei* murine malaria infected Sprague–Dawley rats. *Malar J* 2016; **15**(11): 226.

[15] Huang SS, Chiu CS, Chen HJ, Hou WC, Sheu MJ, Lin YC, et al. Antinociceptive activities and the mechanisms of anti-inflammation of asiatic acid in mice. *Evid Based Complement Alternat Med* 2011; **2011**: 895857.

[16] Ramachandran V, Saravanan R, Senthilraja P. Antidiabetic and antihyperlipidemic activity of asiatic acid in diabetic rats, role of HMG CoA: *in vivo* and in silico approaches. *Phytomedicine* 2014; **21**(3): 225-232.

[17] Guo W, Liu W, Hong S, Liu H, Qian C, Shen Y, et al. Mitochondria-dependent apoptosis of con A-activated T lymphocytes induced by asiatic acid for preventing murine fulminant hepatitis. *PLoS One* 2012; **7**(9): e46018.

[18] Pakdeechote P, Bunbupha S, Kukongviriyapan U, Prachaney P, Khrisanapant W, Kukongviriyapan V. Asiatic acid alleviates hemodynamic and metabolic alterations via restoring eNOS/iNOS expression, oxidative stress, and inflammation in diet-induced metabolic syndrome rats. *Nutrients* 2014; **6**(1): 355-370.

[19] Zhang J, Ai L, Lv T, Jiang X, Liu F. Asiatic acid, a triterpene, inhibits cell proliferation through regulating the expression of focal adhesion kinase in multiple myeloma cells. *Oncol Lett* 2013; **6**(6): 1762-1766.

[20] Ramachandran V, Saravanan R. Asiatic acid prevents lipid peroxidation and improves antioxidant status in rats with streptozotocin-induced diabetes. *J Funct Foods* 2013; **5**(3): 1077-1087.

[21] Yin MC, Lin MC, Mong MC, Lin CY. Bioavailability, distribution, and antioxidative effects of selected triterpenes in mice. *J Agric Food Chem* 2012; **60**(31): 7697-7701.

[22] Yan SL, Yang HT, Lee YJ, Lin CC, Chang MH, Yin MC. Asiatic acid ameliorates hepatic lipid accumulation and insulin resistance in mice consuming a high-fat diet. *J Agric Food Chem* 2014; **62**(20): 4625-4631.

[23] Gumede B, Folb P, Ryffel B. Oral artesunate prevents *Plasmodium berghei* Anka infection in mice. *Parasitol Int* 2003; **52**(1): 53-59.

[24] Matsuoka H, Yoshida S, Hirai M, Ishii A. A rodent malaria, *Plasmodium berghei*, is experimentally transmitted to mice by merely probing of infective mosquito, *Anopheles stephensi*. *Parasitol Int* 2002; **51**(1): 17-23.

[25] Changa KH, Stevenson MM. Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. *Int J Parasitol* 2004; **34**(13–14): 1501-1516.

[26] Brown IN, Phillips RS. Immunity to *Plasmodium berghei* in rats: passive serum transfer and role of the spleen. *Infect Immnun* 1974; **10**(6): 1213-1218.

[27] Zheng XC, Wang SH. Determination of asiatic acid in beagle dog plasma after oral administration of *Centella asiatica* extract by precolumn derivatization RP-HPLC. *J Chromatogr B Analyt Technol Biomed Life Sci* 2008; **877**(5–6): 477-481.

[28] Vincke LH, Bafort J. Results of 2 years of observation of the cyclical transmission of *Plasmodium berghei*. *Ann Soc Belges Med Trop Parasitol Mycol* 1968; **48**(4): 439-454.

[29] Mavondo GA, Mkhwanazi BN, Mabandla MV, Musabayane CT. Asiatic acid influences glucose homeostasis in *P. berghei* murine malaria infected Sprague–Dawley rats. *Afr J Tradit Complement Altern Med* 2016; **13**(5): 91-101.

[30] Sitprija V, Vongsthongsri M, Poshyachinda V, Arthachinta S. Renal failure in malaria: a pathophysiologic study. *Nephron* 1977; **18**(5): 277-287.

[31] Price RN, Simpson JA, Nosten F, Luxemburger C, Hkirjaroen L, ter Kuile F, et al. Factors contributing to anemia after uncomplicated falciparum malaria. *Am J Trop Med Hyg* 2001; **65**(5): 614-622.

[32] Lamikanra AA, Brown D, Potocnik A, Casals-Pascual C, Langhorne J, Roberts DJ. Malarial anemia: of mice and men. *Blood* 2007; **110**(1): 18-28.

[33] Wickramasinghe SN, Abdalla SH. Abdalla, Blood and bone marrow changes in malaria. *Baillieres Best Pract Res Clin Haematol* 2000; **13**(2): 277-299.

[34] Buffet PA, Safeukui I, Milon G, Mercereau-Puijalon O, David PH. Retention of erythrocytes in the spleen: a double-edged process in human malaria. *Curr Opin Hematol* 2009; **16**(3): 157-164.

[35] Phillips RE, Looareesuwan S, Warrell DA, Lee SH, Karbwang J, Warrell MJ, et al. The importance of anaemia in cerebral and uncomplicated falciparum malaria: role of complications, dyserythropoiesis and iron sequestration. *Q J Med* 1986; **58**(227): 305-323.

[36] Rudin W, Quesniaux V, Favre N, Bordmann G. Malaria toxins from *P. chabuadi* chabaudi AS and *P. berghei* ANKA cause dyserythropoiesis in C57BL/6 mice. *Parasitology* 1997; **115**(Pt 5): 467-474.

[37] Wickramsinghe SN, Abdalla SH. Blood and bone marrow changes in malaria. *Baillieres Best Pract Res Clin Haematol* 2000; **13**(2): 277-299.

[38] Martiney JA, Sherry B, Metz CN, Espinoza M, Ferrer AS, Calandra T, et al. Macrophage migration inhibitory factor release by macrophages after ingestion of *Plasmodium chabaudi*-infected erythrocytes: possible role in the pathogenesis of malarial anemia. *Infect Immun* 2000; **68**(4): 2259-2267.

[39] Sengupta P. The laboratory rat: relating its age with human's. *Int J Prev Med* 2013; **4**(6): 624-630.

[40] Sengupta P. A scientific review of age determination for a laboratory rat: how old is it in comparison with human age? *BMIJ* 2011; **2**(2): 81-89.

[41] Andreozzi P, Sarnelli G, Pesce M, Zito FP, D'Alessandro A, Verlezza V, et al. The bitter taste receptor agonist quinine reduces calorie intake and increases the post-prandial release of cholecystokinin in health subjects. *J Neurogastroenterol Motil* 2015; **21**(4): 511-519.

[42] Bevan AP, Christensen JR, Tikerpae J, Smith GD. Chloroquine augments the binding of insulin to its receptor. *Biochem J* 1995; **311**(Pt 1): 787-795.

[43] Cansu DU, Korkmaz C. Hypoglycaemia induced by hydroxychloroquine in a non-diabetic patient treated for RA. *Rheumatol Oxf* 2008; **47**(3): 378-379.

[44] Stevenson MM, Riley EM. Innate immunity to malaria. *Nat Rev Immunol* 2004; **4**(3): 169-180.

[45] van Thien H, Ackermans MT, Dekker E, Thanh Chien VO, Le T, Endert E, et al. Glucose production and gluconeogenesis in adults with cerebral malaria. *QJM* 2001; **94**(12): 709-715.

[46] Xiong Y, Ding H, Xu M, Gao J. Protective effects of asiatic acid on rotenone- or H_2O_2-induced injury in SH-SY5Y cells. *Neurochem Res* 2009; **34**(4): 746-754.

[47] Hsu YL, Kuo PL, Lin LT, Lin CC. Asiatic acid, a triterpene, induces apoptosis and cell cycle arrest through activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways in human breast cancer cells. *J Pharmacol Exp Ther* 2005; **313**(1): 333-344.