



Research Article

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Kidney Function in *P. Berghei*-Infected Sprague-Dawley Rats Following Treatment with Transdermally Delivered *Syzygium-aromaticum* Derived Oleanolic Acid

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Abstract

Impaired renal function has been reported in malaria patients marked by disturbances in electrolytes handling, acute renal failure and increased oxidative stress. Studies in our laboratory indicate that transdermally administered oleanolic acid clears the malaria parasites from *P. berghei*-infected rats. Against this background, the current study investigated the effects of transdermally delivered OA on electrolytes and fluid handling, oxidative stress and selected hormones in *P. berghei*-infected male Sprague-Dawley rats. Renal function was monitored in groups of non-infected and malaria-infected rats following treatment with OA-pectin patch (34mg/kg). The study was divided into pre-treatment (day 0-7), treatment (day 8-12) and post-treatment (day13-21) periods. Animals treated with drug-free pectin and chloroquine (30mg/kg) acted as negative and positive controls respectively.

Infected control animals exhibited reduced urinary sodium (Na⁺) and increased potassium (K⁺) output which was accompanied by hyperkalaemia. The treatment of infected animals with OA-pectin patch increased urinary Na⁺ output, with a concomitant increase in arginine vasopressin. Furthermore, urinary and plasma K⁺ concentrations were restored back to normalcy by day 12 of the study. OA treatment also reduced plasma creatinine concentrations and increased glomerular filtration rate in infected animals. Compared with infected control, OA patch-treated infected animals exhibited significantly reduced malondialdehyde and increased activity of superoxide dismutase and glutathione peroxidase in renal tissues. The current study demonstrates that transdermally delivered OA ameliorates kidney function in malaria-infected animals. These findings are of clinical importance since acute renal failure is a frequent and serious complication associated with *P. falciparum* infection in non-immune patients.

Keywords: Transdermal; Oleanolic acid; Acute renal failure electrolytes; Oxidative stress *Plasmodium Berghei*

Abbreviations: NIC: Non-Infected Control; IC: Infected Control; OCHQ: Orally Administered; Chloroquine; TDOA: Transdermally Administered Oleanolic Acid

Introduction

Malaria infection remains one of the world's leading cause of mortality in many tropical and sub-tropical areas, resulting in 1-3 million deaths annually [1]. This disease is associated with severe life-threatening complications including hypoglycaemia, cerebral malaria and acute renal failure (ARF) [2-5]. The pathogenesis of ARF is multifactorial and involves a complex interaction of immunological, mechanical, humoral factors, and acute phase reactants [6,7]. Cytoadherence of infected red blood cells to the vascular endothelial cells of different host organs, including the kidneys, is reported to alter microcirculation of these organs which ultimately disrupt their physiological functions [8]. Disturbances in electrolyte handling have been reported following malaria infection [9-11]. This hyperkalaemia

is linked to increased haemolysis during malaria infection. Hyponatraemia has long been recognised as a complication of malaria that is mediated through multiple mechanisms including inappropriate production of arginine vasopressin (AVP) [12,13].

Musabayane and colleagues reported increased production of AVP in rodents following oral administration of chloroquine (CHQ), an ant malarial drug. This increase in AVP was associated with increased loss of sodium [14,15]. These findings demonstrate the adverse effects of CHQ treatment on renal function. Hence, there is a need to search and develop novel anti malarial agents with minimal side effects on kidney function. Increased oxidative stress during infection has been implicated in malaria-related acute renal failure. During infection, the Plasmodium parasites

generate large quantities of reactive oxygen species (ROS) [16]. The generation of these ROS is further exacerbated by the host's immune response to the presence of the parasites [17].

These ROS have been reported to exert endothelial damage and renal injury [18,19]. CHQ is a pro-oxidant which exerts its antiplasmodial effects by promoting oxidative stress which subsequently kills the parasite. However, this oxidative stress may also be toxic to the host and could cause tissue damage [20,21]. This further substantiates the need for alternative anti-malarial drugs with minimal side effects. Medicinal plants have long been used to treat multiple ailments including malaria. Preliminary studies in our laboratory indicate that transdermally delivered *Syzygium-Aromaticum* derived-oleanolic acid (OA) possesses antiplasmodial properties in *P. berghei*-infected rats. However, the effects of this transdermally delivered OA on kidney function and malaria-associated oxidative stress are unknown. Hence, the current study was designed to investigate the effects of transdermal application of OA on renal electrolytes handling and oxidative stress in *P. berghei*-infected rats [22].

Materials and Methods

Drug and chemicals

All drugs and chemicals which were of analytical grade quality were sourced from standard pharmaceutical suppliers. Amidated low-methoxyl pectin with a degree of methoxylation (DM) of 23 degree of amidation (DE) of 24 was donated by Dr Hans-Ulrich Endress of Herbstreith and Fox KG, Neuenburg, Germany. Chloroquine diphosphate ($C_{18}H_{26}ClN_3 \cdot 2H_3PO_4$), dimethyl sulphoxide (DMSO), Giemsa stain and May-Grunwald solution (Sigma-Aldrich Chemical Company, St Louis, Missouri, USA); calcium chloride ($CaCl_2$), sodium sulphate (Na_2SO_4), sodium hydroxide (NaOH), potassium dihydrogen phosphate (KH_2PO_4) and 95% ethanol (C_2H_5OH) (Merck Chemicals (PTY) LTD, Johannesburg, South Africa), halothane (Fluorothane®, AstraZeneca Pharmaceuticals (PTY) LTD, Johannesburg, South Africa).

Patch preparation

Amidated pectin hydrogel OA and CHQ-OA matrix patches were prepared using a well-established protocol by Musabayane et al. [23] with slight modifications. Briefly, amidated low methoxyl pectin was dissolved in deionized water (4.4g/110mL) followed by adding OA (1.44g dissolved in DMSO) (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA). However, to prepare the CHQ-OA combination patch, CHQ (5g) and OA, (1.44g dissolved in DMSO) were added together in one beaker, followed by agitation for 30 minutes. Subsequently, eucalyptus oil (1.65mL, Barrs Pharmaceutical Industries cc, Cape Town, South Africa) and vitamin E (1.65mL, Pharmicare Ltd, Johannesburg, South Africa) were added to the mixture and spun for another 1 hour 30 minutes. Following this, an aliquot (11mL) was transferred to a petri dish with a known diameter and frozen at -4 °C for 18 hours. After freezing, 1mL of a 2% $CaCl_2$ solution was added to

allow for cross-linking and formation of the matrix patch. The patches were then stored in a refrigerator at 4 °C until use.

Animals

Male Sprague-Dawley rats (90-120g body weight) bred and maintained at the Biomedical Research Unit, University of KwaZulu-Natal was used in this study. The animals had free access to standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and water, with a 12-hour light/12-hour dark cycle. All animal experimentation was reviewed and approved by the Animal Ethics Committee of the University of KwaZulu-Natal (095/14/Animal).

Induction of malaria

Malaria was induced in male Sprague-Dawley rats with a single intraperitoneal injection of *P. Berghei* parasitized red blood cells (105) [24]. Control animals were injected with phosphate buffered saline vehicle. Successful malaria induction was confirmed by microscopic examination of Giemsa-stained thin smears of the rat tail blood. Percentage parasitaemia of greater than or equal to 20% was considered as a stable malaria state before commencing any experimental procedures.

Application of the hydrogel matrix patch

Rats were shaved on the dorsal region of the neck using the Oster Golden A5 heavy duty single-speed animal clipper (Oster Professional products, McMinnville, Tennessee, United States) 24 hours prior to the application of hydrogel matrix patches. The dermal patches were secured in place with adhesive hydrofilm (Hartman-Congo Inc, Rock Hill, South Carolina, USA) and rat jackets (Braintree, Scientific, Inc, Braintree, Massachusetts, USA) which were adjusted according to the size of the animal.

Short term studies

To evaluate the short-effects of transdermally delivered OA (34mg/kg), dermal patches were topically applied onto the shaved skin area on the back of the neck skin once-off on the first day of the treatment period. The patches were only applied once at 9h00 on day 7 of the study. Oral CHQ (30mg/kg) was administered twice daily for 5 consecutive days, starting on day 8 of the treatment period. Animals treated with a drug-free pectin and CHQ acted as negative and positive controls, respectively. Body weights, amounts of food and water consumed were measured in control and treated animals at 09h00 every 3rd day during the pre-treatment (day 0-7) and post-treatment periods (day 8-12). However following the application of dermal patches, parameters were monitored were monitored on selected days (9,12 and 21).

Urinalysis

Urine volume and urinary concentrations of creatinine, urea, Na^+ , K^+ and Cl^- were determined daily. All measurements were conducted at 09h00. On selected days, blood samples were collected by cardiac puncture into individual pre-cooled heparinized containers for biochemical analysis. Glomerular

filtration rate (GFR), as assessed by creatinine clearance (C_{cr}) was calculated using the standard formulae from measurements of the plasma and urinary concentrations of creatinine and urine flow rate. Urine flow was determined gravimetrically. Na^+ , K^+ , urea and creatinine were analysed using the Beckman Coulter Counter (Synchron CX3 Clinical Systems, Fullerton, California, USA) with commercial diagnostic kits from Beckman Coulter, Dublin Ireland.

Terminal studies

On days 0, 7, 9, 12 and 21, separate groups of animals were sacrificed by exposing to halothane for 3 minutes via a gas anaesthetic chamber (100mg/kg). Blood was collected by cardiac puncture into individual pre-cooled heparinised tubes. Separated plasma was analysed for AVP, aldosterone and electrolytes concentrations. Thereafter, tissues were removed, snap frozen in liquid nitrogen and stored in a Bio Ultra freezer (Snijders Scientific, Tilburg, Netherlands) at $-80^{\circ}C$ until use. Kidney tissues were also collected and stored in formalin for histological analysis.

Effects of treatments on selected hormones

A standard enzymatic method was used to determine plasma AVP and aldosterone concentrations. The assays were performed on Arg8-Vasopressin and aldosterone ELISA Kits, using reagents purchased from the manufacturer (Abcam, Cambridge, Massachusetts, USA). These ELISA Kits are a competitive immunoassay for the quantitative determination of vasopressin and aldosterone in plasma samples. The assay uses a polyclonal antibody-antigen conjugate to bind covalently in a competitive manner with vasopressin/aldosterone in unknown samples. The lower and upper limits of detection were 4 and 923pmol/L, respectively. The intra-assay analytical coefficient of variation ranged from 5.9 to 10.6% and the inter-assay coefficient variation from 6.0 to 8.5%.

Evaluation of oxidative stress

To evaluate and compare the effects of transdermal OA application on oxidative stress in the kidneys of non-infected and malaria infected animals, malondialdehyde (MDA) concentrations were measured. The activities of the antioxidative enzymes, superoxide dismutase (SOD) and that of glutathione reductase (GPx) were assessed as described in the following sections.

MDA

The tissues (50mg) were homogenized in 500 μ L of 0.2% phosphoric acid, followed by centrifugation at 400x G for 10 minutes. Thereafter, 400 μ L of the homogenate was supplemented with 400 μ L 2% phosphoric acid and then separated into two glass tubes. Subsequently, 200 μ L of 7% phosphoric acid was added to each test tube, this was then followed by the addition of 400 μ L of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) into one glass tube (sample test) and 400 μ L of 3mM hydrochloric

acid (HCl) into the second glass tube which served as blank. pH was then lowered to 1.5, by adding 200 μ L of 1MHCl to sample and blank test tubes. The solutions were heated at $100^{\circ}C$ for 15 minutes and allowed to cool to room temperature. Butanol (1.5mL) was added to the cool solutions. The butanol phase (top layer) was then transferred to eppendorf tubes and centrifuged for 6 minutes at 13200xG. The samples were aliquoted into a 96-well microtitre plate in triplicate and the absorbance was read at 532nm (reference λ 600nm) on a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany).

The absorbances from these wavelengths were used to calculate the concentration of MDA using Beer's Law:

$$\text{Concentration of MDA (mM)} = \frac{\text{Average Absorbance}}{\text{Absorption coefficient (156mmol}^{-1}\text{)}}$$

SOD

SOD activity was measured using the Biovision SOD Assay Kit according to manufacturers' instructions (BioVision Research Products, Mountain View, California, USA). Liver and kidney (0.1g) were homogenized in ice-cold 0.1M Tris /HCl (pH 7.4) containing 0.5% Triton X-100, 5mM β - mercaptoethanol (ME) and 0.1mg mL⁻¹ phenylmethanesulfonyl fluoride (PMSF). The tissue homogenate was centrifuged at 14000xG for 5 minutes at $4^{\circ}C$. The supernatant obtained was added to each sample (20 μ L) and blank 2 (20 μ L) well, while blank 1 and blank 3 wells received 20 μ L of H₂O. Thereafter, 200 μ L of working solution was added to each well. Subsequently, dilution buffer (20 μ L) was added to each blank 2 and blank 3 well, while each sample and blank 1 well received enzyme working solution (20 μ L). The solutions were mixed thoroughly for 1 minute before reading the plate. Inhibition activity of SOD was calorimetrically measured on Anthos Venytech-200 Spectrophotometer (Biochrom limited, Cambridge, United Kingdom) after a reaction period of 20minutes at $37^{\circ}C$. SOD activity was calculated as percentage inhibition using the equation:

$$\text{SOD activity (\%inhibition rate)} = \frac{(A_{\text{blank 1}} - A_{\text{blank 3}}) - (A_{\text{sample}} - A_{\text{blank 2}})}{(A_{\text{blank 1}} - A_{\text{blank 3}})}$$

GPx

GPx activity was measured in liver and kidney and heart tissues using the Biovision GPx Assay Kit according to manufacturers' instructions (Bio Vision Research Products, Mountain View, USA). The tissues (0.1g) were homogenized on ice in cold assay buffer (0.2mL) and subsequently centrifuged at 10000 xG for 15 minutes at $4^{\circ}C$. The resultant supernatant (100 μ L) was loaded into a 96-well plate in duplicate. The NADPH standard curve was prepared by diluting the 1mM NADPH standard through a series of concentrations (0, 20,40,60,80, 100nmol per well). The optical density of the standards (OD) was measured at 340nm using Anthos Venytech-200 Spectrophotometer (Biochrom limited, Cambridge, United Kingdom) and the standard curve was constructed from the values obtained.

A reaction mix (90 μ L) containing assay buffer, NADPH, glutathione reeducates and GSH was added to each sample

well and incubated for 15 minutes at room temperature. The OD was then measured (340nm) followed by the addition of cumenhydroperoxide (10µL) and measurement of OD (T1) and another reading following a 5 minute incubation in the dark (25 °C) (T2). GPx activity was calculated using following equation, where the ΔA340nm was used to extrapolate the values of B and Bo from the NADPH standard curve:

$$GPx \text{ activity} = \frac{(B - B_0) \times \text{sample dilution}}{(T_2 - T_1) \times V}$$

Kidney histology

Histological analysis were performed in kidney tissue samples collected from non-infected, infected and *P. berghei*-infected animals treated with OA-pectin hydrogel matrix patch. The tissue samples were fixed in 10% formaldehyde solution and rehydrated in decreasing grades and embedding in paraffin wax. The samples were then sectioned using a microm rotary microtome (Robert-Bosch-Strabe, Walldorf, Baden-Wurttemberg, Germany). Subsequently, the sections were stained with haematoxylin and eosin (H and E) followed by dehydration in increasing grades of ethanol and cleared in xylene. The sections were viewed and captured using Leica light microscope (Leica Biosystems Peterborough Limited, Peterborough, Berkshire, UK).

Statistical analysis

All data were expressed as means±standard error of means (S.E.M.). Statistical comparison of the differences between the control means and experimental groups was performed with Graph Pad In Stat Software (version 5.00, Graph Pad Software, San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. Significant differences were considered at 95% confidence, P<0.05.

Results

Effects of OA-pectin patch on renal electrolyte excretion

The mean daily urinary Na⁺ and K⁺ outputs of non-infected control animals (NIC) remained unchanged throughout the study. *P. berghei*-infected rats (IC) exhibited reduced urinary Na⁺ outputs throughout the study (*IC vs NIC, days 7, 12 and 21; p<0.05, Figure 1A). This reduction in urinary Na⁺ was associated with a continuous increase in urinary K⁺ output (Figure 1B). The loss of loss of K⁺ was also reflected in plasma on day 12 of the study (*IC vs NIC; Table 1). Treatment of animals with oral CHQ (O CHQ) significantly increased (p<0.05) both urinary (Figure 1A) and plasma (Table 1) Na⁺ outputs without affecting chloride (Cl⁻) output. Transdermal application of OA-pectin patch (TD OA) increased (P<0.05) mean daily urinary Na⁺ output (*TD OA vs IC, days 9, 12 and 21; Figure 1A) without inducing any changes in plasma Na⁺ content. Furthermore, urinary (Figure 1B) and plasma K⁺ (4.72±0.68mmol/L Table 1) concentrations were

returned back to normalcy following dermal patch application (TD OA).

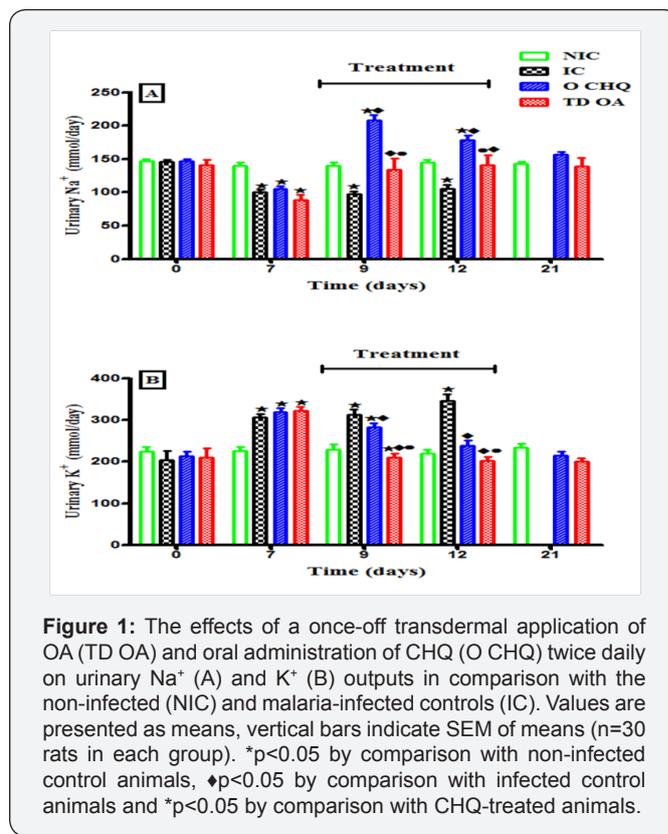


Figure 1: The effects of a once-off transdermal application of OA (TD OA) and oral administration of CHQ (O CHQ) twice daily on urinary Na⁺ (A) and K⁺ (B) outputs in comparison with the non-infected (NIC) and malaria-infected controls (IC). Values are presented as means, vertical bars indicate SEM of means (n=30 rats in each group). *p<0.05 by comparison with non-infected control animals, †p<0.05 by comparison with infected control animals and ‡p<0.05 by comparison with CHQ-treated animals.

Table 1: Comparisons of OA patch and oral CHQ effects on terminal plasma biochemical parameters in malaria-infected animals with non-infected and infected control animals.

Parameter	NIC	IC	O CHQ	TD OA
Na ⁺ (mmol/L)	135.20 ±9.18	102.10 ±7.47*	129.31 ±6.12*‡	137.20 ±9.18‡
K ⁺ (mmol/L)	4.10 ±0.56	6.82 ±0.11*	5.48 ±0.63*	4.72 ±0.68‡
Creatinine (µmol/L)	68.85 ±2.41	98.32 ±3.20*	70.3 ±2.48	69.80 ±1.45
GFR (mL/min/100g)	0.91 ±0.03	0.79 ±0.04	0.85 ±0.01	0.92 ±0.02‡
Kidney mass (g/100g)	0.92 ±0.02	1.15 ±0.01*	1.21 ±0.03*	0.90 ±0.01‡
Water (mL/100g)	15.10 ±2.01	8.10 ±3.10*	7.01 ±2.01*	14.02 ±2.01‡
Food (g/100g)	11.10 ±2.01	6.10 ±2.10*	4.10 ±1.01*‡	11.61 ±1.01‡

Note: The OA pectin patch was applied once on day 7. However, CHQ was administered orally twice daily for 5 consecutive days. Values are presented as means±SEM of means, n=6 rats in each group.

NIC- Non-infected control

IC- infected control

O CHQ- Orally administered chloroquine

TD OA- Transdermally administered oleanolic acid

*p<0.05 by comparison with non-infected control animals.

♦p<0.05 by comparison with infected control animals.

■p<0.05 by comparison with CHQ treated animals.

Effects of OA-pectin patch on renal fluid handling

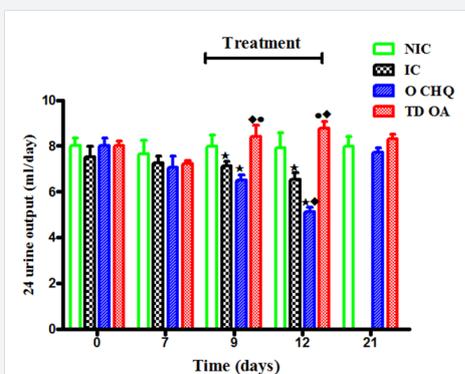


Figure 2: The effects of a once-off transdermal application of OA (TD OA) and oral administration of CHQ (O CHQ) on mean daily urine outputs of *P. berghei*-infected animals in comparison with the non-infected (NIC) and malaria-infected controls (IC). Values are presented as means, vertical bars indicate SEM of means (n=30 rats in each group). *p<0.05 by comparison with non-infected control animals (NIC); ♦p<0.05 by comparison with infected control (IC) animals and ■p<0.05 by comparison with CHQ-treated animals.

Daily mean urine voided was significantly reduced (p<0.05) in infected control animals (IC) throughout the study (IC vs NIC, days 7, 9 and 12; Figure 2). Oral administration of CHQ further decreased the urine output of *P. berghei*-infected animals (♦O CHQ vs IC, days 7, 9 and 12; Figure 2). Interestingly, treatment of infected animals with OA-containing pectin hydrogel matrix patch (TD OA) increased (p<0.05) daily mean urine output back to normalcy (♦TD OA vs IC, days 9, 12 and 21; Figure 2). Furthermore, in comparison to oral CHQ treatment, transdermal application of OA patch was able to achieve daily urine output comparable to the non-infected control in a shorter period of time. (■TD OA vs O CHQ, days 9, 12 and 21, p<0.05; Figure 2).

Effects of oa-pectin patch on plasma biochemical parameters

Biochemical analyses were performed on plasma samples collected from non-infected, infected controls and treated groups on day 12 (last day of the treatment period) of the study. Plasma creatinine concentrations were significantly (p<0.05) elevated in infected untreated (*IC vs NIC, day 12, Table 2) animals by comparison with non-infected control (NIC) animals (Table 2). The OA-pectin hydrogel matrix patch significantly (p<0.05) reduced plasma creatinine in infected animals with a concomitant increase in GFR (♦TD OA vs IC, day 12; Table 2). There were no significant changes in plasma urea concentrations throughout the study; hence the data is not presented. Kidney mass was not altered by transdermal application of OA (♦TD OA vs IC and TD OA vs O CHQ, day 12 Table 2). However, kidney mass

was significantly increased in CHQ treated animals in comparison to the non-infected control as well as OA patch treated group (*■ 1.21±0.03 vs 0.92±0.02 and 0.90±0.01g/100g).

Table 2: Comparison of plasma hormone concentrations in *P. berghei*-infected animals treated with either oral CHQ (O CHQ) or a once-off topical application of OA pectin patch (TD OA).

Group	Time (Days)	AVP (pmol/L)	Aldosterone (nmol/L)
NIC		2.82±0.47	0.81±0.12
IC		2.98±0.62*	0.79±0.05
CHQ (30mg)	12	6.56±0.41*♦	0.79±0.02
	21	3.71±0.02*	0.79±0.11
TD OA (34 mg)	12	2.80±0.14	0.78±0.25
	21	2.79±0.22♦■	0.80±1.18

Note: The OA pectin patch was applied once on the first day of the treatment period. However, CHQ was administered orally twice daily for 5 consecutive days. Values are presented as means±SEM of means, n=6 rats in each group.

NIC- Non-infected control

IC- infected control

O CHQ- Orally administered chloroquine

TD OA- Transdermally administered oleanolic acid

*p<0.05 by comparison with non-infected control animals.

♦p<0.05 by comparison with infected control animals.

■p<0.05 by comparison with CHQ-treated animals.

Effects of OA-pectin patch on terminal hormone measurements

The concentrations of AVP and aldosterone in non-infected control (NIC) animals represent base-line/normal hormonal levels found in plasma samples used (Table 2). Plasma AVP and aldosterone concentrations of the infected control remained unchanged and were comparable to the non-infected control (IC vs NIC, Table 2). Treatment of *P. berghei*-infected animals with oral CHQ (O CHQ) significantly increased plasma AVP concentrations, reaching 6.560.41pmol/L by day 12 of the study (*O CHQ vs NIC, day 12, p<0.05; Table 2). The CHQ-induced increases in AVP lead to increased terminal fluid reabsorption, blood volume and decreased urine output (Table 2 and Figure 2). Interestingly, plasma AVP and aldosterone concentrations of malaria-infected animals were unaltered following a once-off transdermal application of OA-pectin hydrogel matrix patch (TD OA, days 12 and 21vs IC, p<0.05; Table 3), with aldosterone concentrations reaching values that were comparable to baseline/non-infected control (TD OA, days 12 and 21; Table 2).

Effects of OA-pectin patch on oxidative stress

The concentrations of MDA and antioxidative enzymes, SOD and GPx in non-infected control animals represent baseline activity levels found in the kidney tissue used. Untreated *P.*

berghei-infected rats (IC) showed increased ($P < 0.05$) mean MDA concentrations in comparison to the non-infected control (NIC) (6.07 ± 0.62 vs 1.15 ± 0.13 nmol/g protein). Furthermore, SOD and GPx activities were significantly reduced in these animals. Transdermal application of the OA-pectin patch (TD OA) to infected animals reduced MDA levels in the kidney tissues and significantly increased ($p < 0.05$) the activity of oxidative enzymes SOD and GPx activity in comparison to *P. berghei*-infected control animals (TD OA vs IC; Table 3). In comparison to CHQ treatment, OA-pectin patch application was able to achieve MDA levels comparable to the non-infected control (1.21 ± 0.60 vs 7.75 ± 0.41 nmol/g protein).

Effects of OA-pectin patch on kidney histology

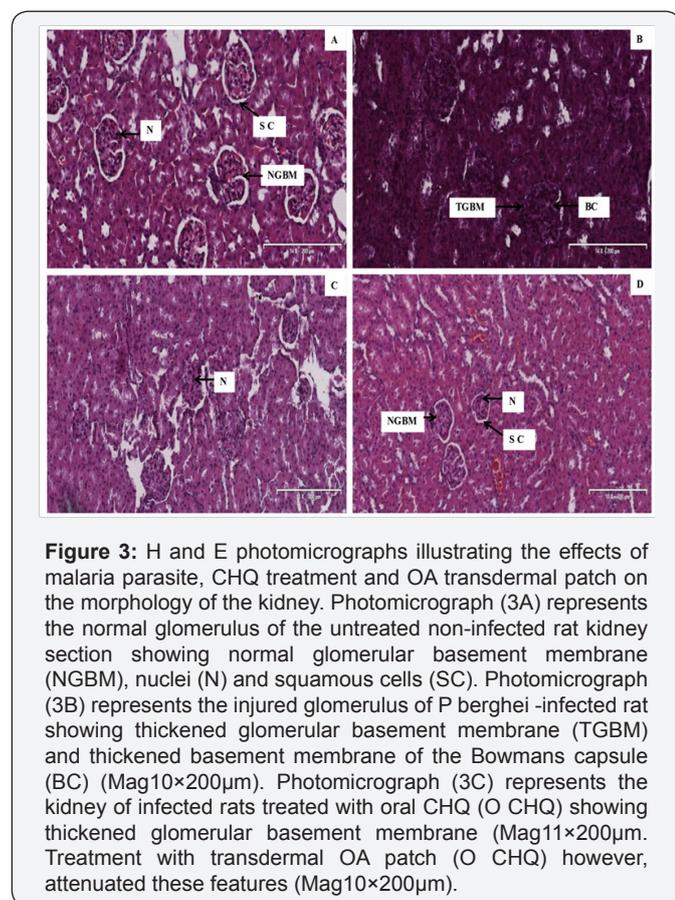


Figure 3: H and E photomicrographs illustrating the effects of malaria parasite, CHQ treatment and OA transdermal patch on the morphology of the kidney. Photomicrograph (3A) represents the normal glomerulus of the untreated non-infected rat kidney section showing normal glomerular basement membrane (NGBM), nuclei (N) and squamous cells (SC). Photomicrograph (3B) represents the injured glomerulus of *P. berghei*-infected rat showing thickened glomerular basement membrane (TGBM) and thickened basement membrane of the Bowman's capsule (BC) (Mag $10 \times 200 \mu\text{m}$). Photomicrograph (3C) represents the kidney of infected rats treated with oral CHQ (O CHQ) showing thickened glomerular basement membrane (Mag $11 \times 200 \mu\text{m}$). Treatment with transdermal OA patch (O CHQ) however, attenuated these features (Mag $10 \times 200 \mu\text{m}$).

Figure 3A shows the normal section of the kidney of the non-treated group, illustrating normal glomerular basement membrane (NGBM), nuclei (N) and squamous cells (SC). Compared to the untreated non-infected control rats (NIC) Figure 3A, *P. berghei*-infected rats (IC) Figure 3B showed thickened glomerular basement membrane (TGBM) and thickened basement membrane of the Bowman's capsule (BC). Oral CHQ (O CHQ) Figure 3C treated rats showed thickened glomerular basement membrane. Treatment with OA transdermal patch (TD OA) Figure 3D however, attenuated these features when compared with the untreated *P. berghei*-infected rats Figure 3B and oral CHQ.

Discussion

The current study evaluated the effects of transdermally delivered OA on renal function of *P. berghei*-infected animal models. The results indicate that OA-pectin patch delivers sustained OA doses which are able to improve oxidative stress and ameliorate kidney function in malaria-infected animals. These findings are of clinical importance since acute renal failure is a frequent and serious complication associated with *P. falciparum* infection in non-immune patients [6,7,25]. Furthermore, these findings offer a novel alternative for malaria treatment which not only clears malaria parasites but also improves renal function of infected animals. Oleanolic acid is a pentacyclic triterpenoid with multiple pharmacological actions [26-29]. However, orally administered OA is susceptible to metabolism by cytochrome P 450 isoenzymes in the intestinal tract, resulting in low bioavailability of the drug [30].

The decreased bioavailability of OA has led to the limited application of this drug. In the current study, we were able to deliver OA through the skin using a pectin-OA patch. The transdermal route of delivery avoids hepatic and intestinal metabolism of OA by delivering OA directly into the systemic circulation and allow for the use of low drug concentrations. *P. berghei*-infected control animals exhibited significantly reduced urinary and plasma Na^+ concentrations. Malaria-related renal electrolytes handling remains unclear and incompletely understood. The reduction in plasma Na^+ concentrations has been linked to multiple mechanisms including inappropriate production of antidiuretic hormone, depletion Na^+ and unexplained resetting of the osmoreceptors [12,31,32]. In the current study, there were no significant changes in plasma AVP concentrations in the infected control animals, suggesting a possibility of other sodium depleting mechanisms.

This data further demonstrates that there are indeed disturbances in renal function during infection with malaria. Treatment of infected animals with OA containing dermal patches increased both urinary and plasma Na^+ concentrations to values comparable to the non-infected control (which representative of the normal values of Na^+ in non-infected animals). The ability of transdermally administered OA to restore urinary Na^+ output and ameliorate hyponatraemia in infected animals is demonstrative of the potency of this formulation to ameliorate kidney function in malaria-infected animals. Orally administered CHQ has been the mainstay therapy in many malaria endemic areas. Treatment with CHQ was associated with an increase in urinary Na^+ excretion. The CHQ-induced natriuresis has been linked to increased production of the antidiuretic hormone, AVP. Indeed, there was a significant increase in plasma AVP concentrations following treatment with CHQ (Table 3).

These results are in agreement with previous findings which attributed the CHQ-related natriuretic effects to the action of AVP on V1 receptors [14], where AVP exerts pressor effects

causing an increase in Na⁺ excretion without changing the urine flow rate. In summary, the current data demonstrates that the treatment of malaria-infected animals with OA-pectin patch improves renal electrolyte handling. Hyperkalaemia is one of the factors associated with acute renal failure (ARF) in severe malaria infections [11,33]. Urinary K⁺ output was increased in all untreated *P. berghei*-infected animals on day 7 of the treatment period Figure 1B. The increase in urinary K⁺ output was associated with an increase in plasma K⁺ concentrations.

We speculate that the observed hyperkalaemia is mainly due to uncontrolled intravascular haemolysis associated with malaria infection [34]. The release of potassium from ruptured RBCs in turn, causes an increase in plasma K⁺ concentrations (Table 1). Hyperkalaemia was exacerbated by oral CHQ treatment. CHQ exerts its anti malarial effects by causing cell lysis which could further increase plasma K⁺ concentrations. Following treatment with OA-pectin patch, there was a reduction in daily urinary K⁺ output. Plasma K⁺ concentrations were also restored by day 12 of the study (Table 1). The ability of OA treatment to restore both plasma and urine K⁺ output might be linked to the clearance of the malaria parasites during treatment. The clearance of parasitaemia from circulation in turn, eliminates the uncontrolled destruction of RBCs. Hence, less potassium is released from red blood cells.

The elevated plasma creatinine concentrations observed in the infected control animals is suggestive of inappropriate filtering of the kidney. ARF is associated with increased plasma creatinine concentrations and more prevalent in adults than children [35]. This is further substantiated by Idonije et al. [36] who reported increased plasma creatinine concentrations in malaria-infected patients. The observed increase in plasma creatinine could be ascribed to the sequestration of the parasites into the renal microvasculature bed which may lead to ischemia [4,37]. The elevated plasma creatinine concentrations were accompanied by a significant decrease in GFR. During malaria infection, there is increased mechanical obstruction by parasitized red blood cells (PRBCs), exaggerated host immune response mediated through cytokines, reactive oxygen and nitrogen species, immune complex deposition and disturbances in the renal microcirculation which often results in glomerular cell proliferation, basement membrane thickening [4,34].

These structural changes lead to functional changes at a cellular level [38]. The decrease in GRF that was observed in the current study may be attributed to the enlargement of the glomerular basement membrane (TGBM) and basement membrane of the Bowmans capsule (BC) that was observed in histological studies. The decrease in plasma creatinine concentrations following treatment with the OA-pectin patch could be due to the elimination of the parasites from the systemic circulation. As a result, there was no Cytoadherence of parasitized red blood to the vascular endothelial cells of the kidney which could ultimately impair renal function [8].

Table 3: Comparison of MDA concentration, activities of SOD and GPx in kidney tissues of malaria-infected animals treated with either a once-off transdermal application of a OA-pectin patch or orally administered CHQ (O CHQ) with non-infected (NIC) and infected control (IC) animals.

Group	MDA (nmol/g protein)	Activity (nmol/min mL/g protein)	
		SOD	GPx
NIC	1.15±0.13	19.64±1.89	4.40±0.32
IC	6.07±0.62*	5.31±0.66 [‡]	0.36±0.05*
O CHQ	7.75±0.41*	1.87±0.04 [‡]	0.19±0.02*
TD OA	1.21±0.60 ^{◆■}	24.03±0.61 ^{◆◆*}	8.01±0.10 ^{◆◆*}

■p<0.05 by comparison with non-infected control animals

◆p<0.05 by comparison with infected control animals

*p<0.05 by comparison with oral CHQ treated animals.

NIC- Non-infected control

IC- infected control

O CHQ- Orally administered chloroquine

TD OA- Transdermally administered oleanolic acid

Furthermore, OA-patch treatment increased GFR of infected animals. The improved GFR following OA-patch application might be linked to the ability of OA to restore the histological features of the TGBM and basement membrane of the Bowmans capsule in kidneys. This increase in GFR demonstrates the ability of OA to improve kidney function of malaria-infected animals. Our data indicated that the increased MDA (a marker for lipid peroxidation) concentrations in kidney tissues of untreated infected rats were restored back to normalcy after treatment with OA containing patches (Table 3). The reduction in MDA concentrations could be due to the clearance of the malaria parasites and increased activities of SOD and GPx, which in turn improved the antioxidant status in the kidney tissues. The degradation of host's haemoglobin by the Plasmodium parasites is thought to be a major cause of oxidative during malaria [39].

During infection, there is increased production of cytokines and reactive oxygen species (ROS), which in turn cause increased lipid production, nitric oxide, inflammation and depletion of antioxidant defence in some tissues including the kidney [40-42]. In the current study inflammation of the kidneys was confirmed by histological studies which showed the thickening of the glomerular basement membrane (TGBM) and basement membrane of the Bowmans capsule (BC). This thickening of the glomerular basement membrane (TGBM) and basement membrane of the Bowmans capsule may be responsible for the decreased GFR that was observed in untreated *P. berghei*-infected rats. OA-patch treatment was able to attenuate the inflammation in the kidney tissues and increase the GFR of infected rats. OA has been shown to be a potent anti-inflammatory agent during inflammation [43].

Treatment with OA-pectin patch significantly improved the antioxidant status of infected animals by comparison to CHQ

treatment. CHQ is a schizonticidal and gametocytocidal drug which exerts its antimalarial activity by increasing oxidative stress [22]. Indeed, there was an increase in MDA and a reduction in antioxidant status in the kidney tissues of infected animals treated with CHQ which further demonstrates the pro-oxidant properties of this drug. The ability of the OA patch to alleviate oxidative stress in kidney tissues is further substantiated by the attenuation of defects in the microscopic structures of the kidney. Furthermore, the kidney mass of these animals was reduced back to normal after treatment with transdermal OA. The amelioration of oxidative stress by OA-pectin patch indicates that transdermally administered OA improves renal function of infected animals [44].

The data presented in the current study shows that transdermal delivery of OA improves renal function in malaria-infected animals by, ameliorating electrolytes handling, oxidative stress and inflammation. These findings are of great importance since acute renal failure is a frequent and serious complication which can cause mortality in malaria patients. The formulation presented in the study offers an alternative antimalarial drug which clears malaria parasites and improves kidney function in infected animals.

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