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Effect of Captopril on Some Peptides of Renin Angiotensin System (RAS) in *Plasmodium berghei*-Infected Mice

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Authors' contributions

This work was carried out in collaboration among all authors. Author BK designed the study. Author MKA wrote the protocol. Author URM carried out the laboratory work and performed the statistical analysis. Author AMA wrote the first draft of the manuscript. Author MB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: There are conflicting reports on the effect of peptides of the Renin Angiotensin System (RAS) on the pathogenesis of malaria infection. Captopril is a common antihypertensive drug that inhibits angiotensin-converting enzyme (ACE), a critical peptide of RAS, and its effect on the pathological process of malaria is just beginning to unfold. This study was carried out to investigate the effect of captopril on parasitemia, ACE, angiotensin II, and angiotensin II type 1 receptor (AT_1R) levels in *Plasmodium berghei*-infected mice.
Methodology: Twenty-five mice divided into: Group 1 (control), Group 2 (Malaria control), Groups 3, 4 and 5 (Treated with 0.03 mg/kg lonart, 0.03 and 0.09 mg/kg captopril, respectively) were treated for 14 days. On the 15th day, they were sacrificed to obtain the blood and kidneys. The concentration

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of ACE and Ang II in serum and kidneys; and expression of ACE and AT_1R in kidneys were assayed using standard protocols.

Results: There was a significant decrease (P<0.05) in parasitemia in all treated mice compared to malaria control mice, and by the 14th day, the parasitemia level in mice given 0.09 mg/kg captopril and 0.03 mg/kg lonart was not different (P>0.05) from control. The ACE and angiotensin II levels in serum and kidneys of malaria-infected mice increased significantly (P<0.05), but decreased (P<0.05) when given 0.03 mg/kg lonart, and both doses of captopril. With respect to gene expression, the ACE and AT_1R mRNA were upregulated (P<0.05) in the kidneys of diseased mice, but treatment with captopril resulted in a dose-dependent decrease (P<0.05).

Conclusion: Captopril inhibits *Plasmodium* parasite; and this may be due to its ability to down-regulate ACE and AT_1R expression.

Keywords: Malaria; captopril; angiotensin-converting enzyme; Plasmodium berghei; angiotensin II type 1 receptor.

1. INTRODUCTION

Malaria is an infectious disease characterized by the invasion of red blood cells by *Plasmodium* parasite, the most severe of which is related to *Plasmodium falciparum*, responsible for majority of deaths [1]. About half of the world's population (3.3 billion) is affected by malaria [1]. According to WHO [2], in 2017, there were 435,000 deaths due to malaria, 93% of which occurred in Africa, with 61% of them being children below 5 years. Malaria comes 3rd, after pneumonia and diarrheal disease as the leading cause of death in children under five years worldwide [2]. There are more cases and deaths due to malaria in Nigeria than any country in the world, with 97% of the population at risk. It is estimated that there are 100 million cases of malaria, causing over 300,000 deaths per year in Nigeria, which is higher than 215,000 deaths from HIV/AIDS in the country [1,2].

The peptides of the renin angiotensin system (RAS), via G-protein coupled receptors (GPCRs) are involved in the regulation of vascular volume and blood pressure in the kidneys and cardiovascular system. Angiotensin converting enzyme (ACE) (EC3.4.15.1), a zinc metallopeptide of RAS, hydrolyzes the carboxyl terminal dipeptide of angiotensin I and bradykinin to form angiotensin II (Ang II) [3]. It is found in various body organs, but predominantly the kidney epithelium [4]. Captopril is an ACE inhibitor used for the treatment of heart-related ailments, like hypertension, cardiac failure, diabetic nephropathy, myocardiac infarction etc [5]. ACE inhibitors competitively inhibit ACE, resulting in low levels of angiotensin II and bradykinin, causing dilation of the arteries and veins and consequently low blood pressure. Also, inhibition of angiotensin II decreases aldosterone secretion causing a decrease in water and sodium reabsorption and a reduction in extracellular volume [6]. Ang II is a pro-inflammatory and pro-fibrotic agent, which contributes to organ damage in disease [7,8,9,10,11,12,13], but its effect on the pathogenesis of malaria and other parasitic diseases is only just evolving.

Recent reports implicate RAS in the pathogenesis of malaria [14,15,16], although receptors of RAS peptides are yet to be described in the genome of malaria parasite. Ang II and other peptides within RAS have been shown to impair the erythrocytic cycle of the malaria parasite, reduce its growth *in vitro* [14,15], delay cerebral malaria (CM) and decrease brain hemorrhage incidence in *P. berghei*-infected mice [16]. Also, synthesized analogues of this peptide have been demonstrated to possess antiplasmodial activities. These analogues are important so as to avoid the biological effects of angiotensin peptides on vascular and renal organs [14,15,16,17,18,19]. Studies suggest that the "protective" effect of Ang II may be attributed to mechanisms that affect host susceptibility and/or parasite development at the time of infection [14,15,16].

These results differ from more recent reports showing that Ang-(1-7) was responsible for amelioration of the disease [20,21]. Silva-Filho et al. [7,22] reported that losartan, an AT1R inhibitor, and captopril, an inhibitor of ACE reduces parasitemia, helps infected mice survive and prevents development of CM. They reported that high Ang II level causes severe malaria, by activating angiotensin II type 1 receptor (AT_1R), which stimulates inflammation and activates endothelial cells. Also, Gallego-Delgado and

Rodriguez [23] associated ACE and ACE2 polymorphisms and increased Ang II levels to malaria severity in adults with an African genetic background, while it confers protection in childhood. The pharmacological blockage of AT_1R and reduction of Ang II level protected mice against CM and increased survival [24]. Also, inhibitors of Ang II and its receptors were shown to provide protection against malaria [7,22,25]. This apparently does not conform to reports that a high Ang II level prevents severe malaria. Thus, for better understanding of RAS and malaria pathogenesis, research on the effect of RAS inhibitors on the expression of RAS peptides in malaria infection needs to be investigated, as it has been shown that regulation of the expression and activity of RAS components play significant roles in the pathogenesis of the disease [14,15,25]. The present study aims to determine the effect of an ACE inhibitor (captopril) on parasitaemia, levels and expression of ACE and AT_1R in mice infected with *P. berghei*, to provide insights to the pathway(s) mediating their effect.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Ang-II and ACE ELISA kits (Elabscience Biotechnology Company, Ltd, China), Lonart Arthemeter+lumefantrin (BLISS GVS Pharma Ltd, India), RNA Isolation kits (Thermo Fisher, USA). All other reagents were of analytical grade.

2.2 Experimental Design

Twenty-five (25) apparently healthy mice weighing between 24 to 34 g were used for the study. The animals were obtained from the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State. All animals were maintained 5 per cage, fed standard animal feed and water and maintained under standard conditions.

Doses were chosen after carrying out a pilot study to determine the effective oral dose of captopril that would significantly reduce parasitemia and decrease mortality.

The animals stayed two weeks to acclimatize to the environment before commencement of experiment. They were randomly divided as follows;

Group I: Control (uninfected, untreated)

Group II: Malaria control (malaria infected, untreated)

Group III: Standard control (malaria infected, and treated with 0.03 mg/kg Lonart)

Group IV: Malaria-infected and administered 0.03 mg/kg captopril

Group V: Malaria-infected and administered with 0.09 mg/kg captopril

Mice were given the drugs orally everyday for fourteen days and parasite level determined using microscopic method every other day.

All experimental protocols were approved and conducted with strict adherence to guidelines and procedures of the Institutional Animal Care and Use Committee of Bayero University, Kano.

2.3 Induction and Estimation of Parasitaemia

The Department of Parasitology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Kaduna State, Nigeria, provided the ANKA strain of *P. berghei*. The day mice were intraperitoneally inoculated with 0.2 ml blood containing 1×10^7-parasitized red blood cells was taken as day zero. Parasitemia levels of newly inoculated mice were monitored every other day [26].

Microscopic diagnosis for malaria was carried out using Giemsa stain as described by Cheesbrough [27]. Blood was collected from mice tail and a thin film made on a grease-free slide. The film was allowed to dry at room temperature before adding giemsa stain and allowed to stand for 10 minutes. The slide was washed with distilled water and allowed to stand for 5 minutes before cleaning with a damp cotton wool in methylated spirit and examined under microscope after drying. Parasitemia was determined as percentage of parasitized red blood cells to total red blood cells counted, while, percentage inhibition of parasitemia by the drug was determined thus:

% Inhibition = [Parasitemia in control (uninfected) mice – Parasitemia in treated group)] X 100/ Parasitemia of control (uninfected)

2.4 Preparation of Tissue Homogenate

On the 15th day, blood was collected from mice sacrificed by anaesthesia. For serum determination of ACE and Ang II level, blood was centrifuged at 1000xg at 4°C for 10 minutes. For

determination of renal ACE and Ang II level, some portions of kidneys collected after dissection was macerated in Phosphate buffer (pH 7) on ice, and homogenates centrifuged for 5 minutes at 1000xg to obtain the supernatant, which was preserved at 4°C. For RNA analysis, another portion was washed in saline, frozen immediately in liquid nitrogen and stored at -40°C.

2.5 Determination of Angiotensin II and Angiotensin Converting Enzyme Concentration

This was determined using a sandwiched ELISA protocol according to the manufacturer's instructions (Elabscience Biotechnology Company, Ltd, China), where ELISA plates pre-coated with antibodies specific to ACE (for determination of ACE concentration) or Ang II (for determination of Ang II concentration) is provided. Briefly, 100 µL each of the standard, blank, and samples (homogenate and serum) were pipetted into different wells, incubated at 37ºC for 90 minutes, and aspirated. This was followed immediately by addition of biotinylated detection antibody (100 µL), sealed and tapped to mix contents and incubated for 1 hour at 37°C. After incubation the wells were aspirated, properly washed and horseradish peroxidase (100 µL) added. This was incubated at 37°C for 30 minutes, washed again, before adding the substrate (90 µL). Incubation with the substrate was done at 37°C for 15 minutes, after which the reaction was stopped using a stop solution, and the optical density (OD value) determined with a micro-plate reader at 450 nm. The OD value is proportional to the concentration of ACE. The concentration of the ACE of each sample was determined by comparing the OD of the samples to a standard curve [28].

2.6 Determination of ACE and AT1R Expression

2.6.1 Isolation of total RNA from kidneys

Total RNA was isolated from kidney tissues according to manufacturer's instructions (Thermo Fisher, USA). Briefly, about 50 mg of the frozen tissue was disrupted in liquid nitrogen by grinding into powder using a mortar and pestle. Lysis buffer (VRX, pH 7) was added to the powder, mixed immediately and homogenized. The mixture was centrifuged at 1000xg for 10 minutes and supernatant was transferred into an RNase-free microcentrifuge tube.

2.6.2 RNA quantification, integrity and purity

Total RNA was quantified using a Nano Drop 1000 spectrophotometer and the integrity was determined using 2% agarose gel electrophoresis. Briefly, 100 ml of Tris-acetic-EDTA buffer (pH 7, 24.2 g Tris, 5.71 g glacial acetic acid and 0.5 mM EDTA) was added to 2 g of agarose in a 250 mL conical flask. The suspension was heated in the microwave oven for 5 minutes to melt the agarose, and ethidium bromide added before pouring into the agarose gel rack with the cartridge well placed. The gel was then loaded with samples after solidification, placed in the electrophoresis compartment and TAE buffer (pH 7) was added. The set up was then connected to a power pack and ran for 45 minutes at 10 V/cm before viewing using the gel documentation machine.

2.6.3 Primer design

The DNA sequence of the genes of interest (ACE) and (Ang II) were obtained from NCBI (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide), before designing appropriate primers. Care was taken to avoid non-coding regions within the sequences. Also, a BLAST search was run on primers to be sure of their non-annealing to other targets. Primer sequences were as follows: ATR1: Forward primer 5'-CCATCACCAGATCAAGTGCA-3', Reverse primer 5'-TGGGGCAG TCATCTTGAATTCT-3'; ACE: Forward primer 5'-CCCA TCTGCTAGGGAACATGT-3', Reverse primer5'-GGTGTCCATCCCTGCTTTATCA-3'; and GADPH: Forward primer 5'-GGAGA AA CCTGCCAAGTATG-3', Reverse primer5'-AGG AGACAACCTGGTCCTCA-3'. PCR Conditions for ACE, AT_1R and GADPH was 30 cycles of initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, elongation at 72°C at 30 seconds and final elongation at 72°C for 5 minutes.

2.6.4 Real-time reverse transcriptase polymerase chain reaction (RTRT-PCR)

Complementary DNA was synthesized from the RNA and reverse transcribed using cycle script RT premix kit (Bioneer Corporation) which contains oligo DT primers. Briefly, 15 µl of RNA was added into the PCR microtube and 5 µl of RNase free water was added directly and centrifuged at maximum speed for 1 minute. The tube was inserted into the PCR machine and the

following condition was set for the cDNA synthesis: 50°C for 1 hour and 94°C for 5 minutes. For RTRT-PCR, a 20 μl reaction tube comprising 10 μL of master mix (containing all PCR optimizers), 0.5 μL forward and 0.5 μL reverse primers, 7μl of water and 2 μl of sample (cDNA) was prepared and real time polymerase chain reactions for ACE, AT_1R and GADPH was then run after setting appropriate conditions on the RT-PCR machine [29]. The relative quantification of ACE and AT_1R expression was normalized to GADPH and calculated with the Pfaffl method using the Relative Expression Software Tool (REST, http://www.rest.gene-quantification).

2.7 Statistical Analysis

Univariate and bivariate analysis using SPSS 17 software program was utilized. The mean±SD of all numerical values was tested by ANOVA test, and *P* values < 0.05 was considered statistically significant.

3. RESULTS

The percentage (%) parasitemia of treated and control mice are presented in Fig. 1. From the results percentage parasitemia of all mice before the induction (day zero) was zero, and on day 2 of treatment, it increased significantly (P<0.05) in all mice except the control group. The parasitemia level of malaria control mice (4.82±0.04%) was higher (P<0.05) than all groups (Fig. 1).

High parasitemia increased (P<0.05) serum and renal ACE activity and concentration, but these decreased (P<0.05) after treating mice with 0.03 mg/kg lonart, 0.03 mg/kg and 0.09 mg/kg captopril (Tables 1 and 2). The concentration of Ang II in serum and kidneys of *P. berghei*-infected mice was higher (P<0.05) in malaria control mice than untreated control mice, but decreased (P<0.05) in mice given 0.03 mg/kg lonart, 0.03 mg/kg captopril and 0.09 mg/kg captopril. The decrease in serum and renal Ang II concentration of mice given 0.03 mg/kg captopril was lower (P<0.05) than mice given 0.09 mg/kg captopril, showing a dose-dependent effect.

The real time quantitative RT-PCR results showed that ACE mRNA level was higher (P<0.05) in the kidneys of malaria control (23.53±4.26) than control (1.06±0.02) mice. After treatment with 0.09 mg/kg captopril, the mRNA reduced (0.81±0.14) and was significantly (P<0.05) different from that of mice given 0.03 mg/kg lonart (6.69±2.01) and 0.03 mg/kg captopril (3.50±1.36). The ACE mRNA of mice given 0.09 mg/kg captopril (0.81±0.14) and those in the control group (1.06±0.02) was not different (P>0.05) (Fig. 2).

Figs. 3 shows that AT_IR mRNA level also increased (P<0.05) in the kidneys of malaria control (30.30±4.47) compared to control (1.02±0.03) mice, but treatment with 0.09 mg/kg captopril, reduced (P<0.05) it to 2.19±0.65 folds, further lower (P<0.05) than mice given 0.03 mg/kg lonart (8.50±2.01) and 0.03 mg/kg captopril (7.50±1.61), showing a dose-dependent response.

4. DISCUSSION

Recently the world's attention has been drawn to malaria control and financial support extended to research focused on drug development, antimalarial vaccines and vector control [30]. The ability to sequence genomes of malaria parasites [31], the vector *Anopheles gambiae* [32], and developing genetically modified mosquitoes that are parasite-resistant [33] serve as keystones in the fight against malaria control [34].

Table 1. Renal and serum concentrations of angiotensin converting enzyme in *P. berghei*-infected mice

GROUP	ACE concentration (ng/mL)	
	Serum	Kidneys
Control	$0.19±0.02^a$	$0.50±0.35^a$
Malaria control	$3.79±0.60^d$	$3.78±0.73^d$
Standard drug (lonart) (0.03mg/kg)	$0.19±0.03^a$	$0.49±0.11^a$
Captopril (0.03mg/kg)	$0.15±0.01^c$	$0.19±0.48^c$
Captopril (0.09mg/kg)	$0.11±0.01^b$	$0.15±0.05^b$

Values with different superscripts in a column are significantly different at p<0.05

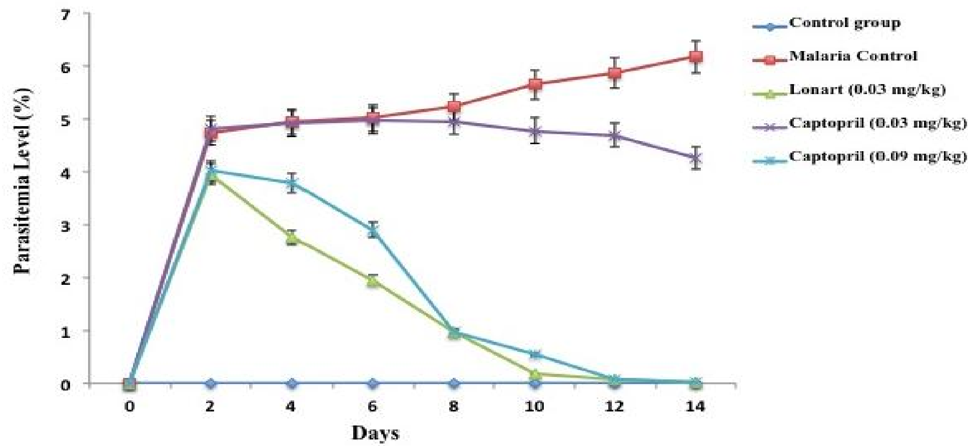

Fig. 1. Percentage parasitemia before and after administration of drugs in treated and untreated *P. berghei*-infected mice

Table 2. Renal and serum concentration of angiotensin II in *P. berghei*-infected mice

Group	ANG II level (ng/mL)	
	Serum	**Kidneys**
Control	1.39±0.09[a]	2.15±0.20[a]
Malaria control	4.93±0.51[b]	5.61±0.60[b]
Standard drug (lonart) (0.03mg/kg)	1.56±0.56[a]	2.10±0.45[a]
Captopril (0.03mg/kg)	3.39±0.34[c]	3.96±0.10[c]
Captopril (0.09mg/kg)	1.57±0.15[a]	2.08±0.93[a]

Values with different superscripts in a column are significantly different at p<0.05

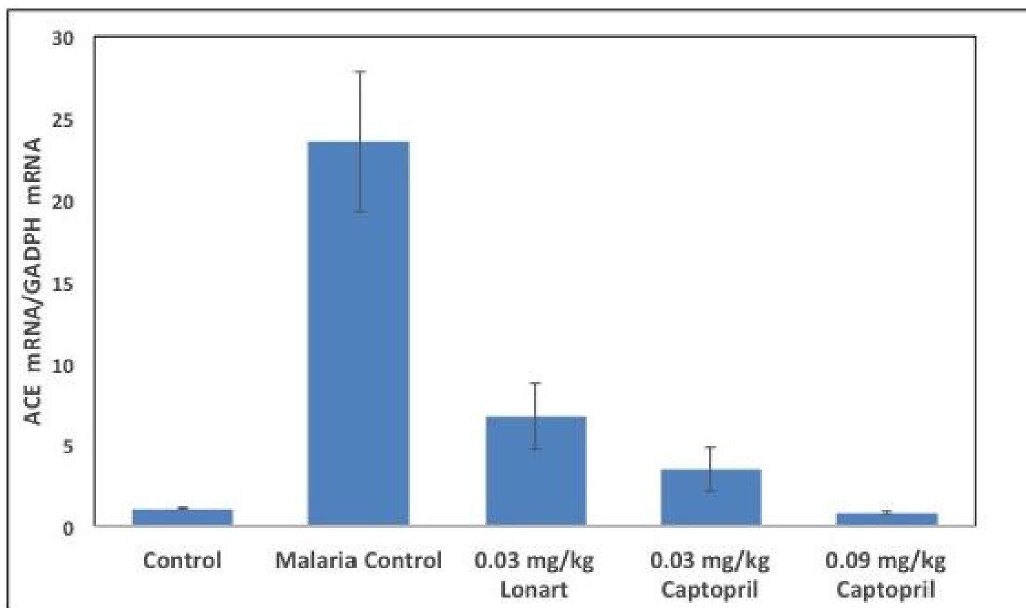

Fig. 2. Effect of captopril on the expression of angiotensin converting enzyme in kidneys of *P. berghei*-infected mice

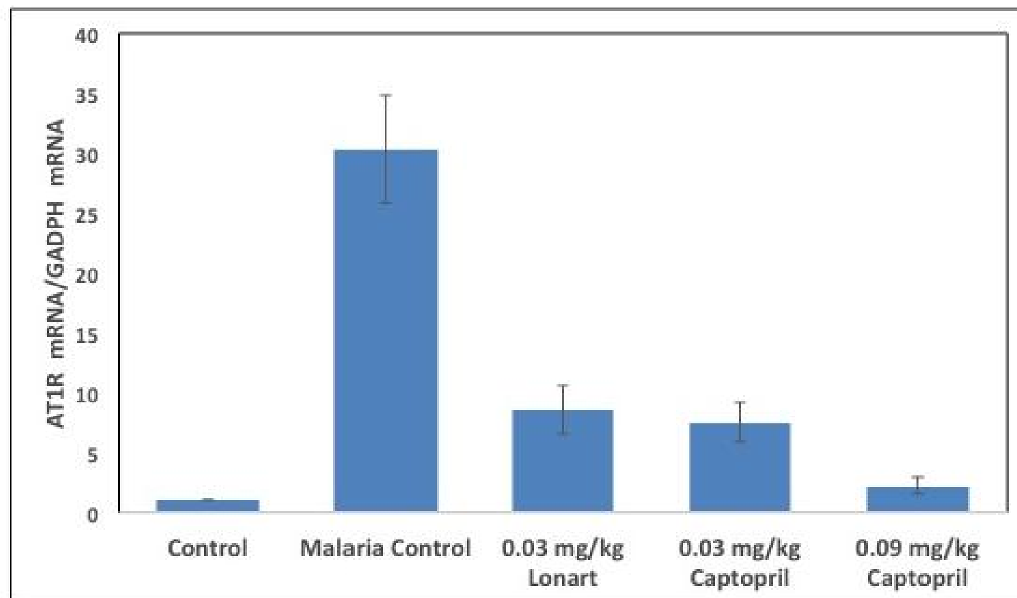

Fig. 3. Effect of captopril on the expression of angiotensin type 1 receptor in kidneys of *P. berghei*-infected mice

This study was done to explore the hypothesis that captopril, an ACE inhibitor has a positive effect on malaria, by monitoring its effect on parasitemia in mice infected with *P. berghei*. Also, its effect on the activity of ACE and Ang II level as well as ACE and AT_1R expression was determined. Initial results revealed that treatment with 0.09 mg/kg captopril reduced parasite level better than 0.03 mg/kg captopril, indicating a dose-dependent effect. Also, after 14 days of treatment, parasitemia of mice treated with 0.09 mg/kg captopril was not different (P>0.05) from control. This result correlate with a study by Silva-Filho et al. [7] who reported that captopril reduced parasitemia, increased survival time and prevented development of CM. The study attributed this to the inhibition of ACE by captopril, which causes accumulation of Ang I and results in its conversion to Ang 1-7,known to reduce the *in vitro* invasion of *P. Falciparum* [18]. On the other hand, the findings of this study disagrees with Maciel et al. [14] and Saraiva et al. [15] that elevated levels of angiotensin II and its related peptides impaired the red blood cell stage of *the* parasite, thus, reducing its growth *in vitro*. They also suggested that Ang II affects the salivary gland membrane to reduce accumulation of sporozoites. Another study reported that Ang II treatment of mice infected with *P. berghei* reduced blood parasitemia and delayed CM [16].

To corroborate these studies associating elevated Ang II to reduced malaria infection, genetic studies have shown that two polymorphisms of ACE and ACE2 known to cause elevated Ang II are associated with low malaria incidence within some populations [35,36]. However, in a recent study, same researchers [19] verified that blockage of AT_1R and reduction of Ang II levels prevented CM and increased survival in mice. According to Saraiva et al. [15] and Silva et al. [20,21], the reduction in parasitemia and amelioration of CM observed in mice is a result of the fast metabolism of accumulated Ang II to Ang-(1-7), and not the high Ang II levels, because high Ang II level stimulates *Plasmodium*-specific $CD8^+$ T cell, the most important effector cell in experimental CM [25]. They demonstrated that Ang-(1-7) decreased parasitemia by causing the impairment of red blood cell stage development of *P. falciparum*. This reduces protein kinase A (PKA) activity mediated via Mas receptor, and induces vasodilation through bradykinin amplification, cGMP synthesis stimulation and inhibition of norepinephrine. They also showed that captopril, an ACE inhibitor, mimicked the Ang-(1-7) inhibitory activity, as captopril inhibition of ACE causes accumulation of Ang I, which is converted to Ang-(1-7) mediating the same effect.

The high level of parasitemia led to significantly ($P<0.05$) high levels of renal and serum Ang II and ACE levels, while treatment with 0.03 mg/kg lonart, 0.03 and 0.09 mg/kg captopril caused a significant ($P<0.05$) decrease after 14 days treatment. It is a known fact that high parasitemia is responsible for the lethality and severity of malaria, and from the present study, it caused elevation of ACE and Ang II levels. This shows that the inhibition of ACE by captopril, as observed in this study decreases Ang II levels in treated mice, causing accumulation of Ang I, and its subsequent conversion to Ang (1-7) [14,15,20,24]. The low ACE and Ang II in mice given the antimalarial drug, Lonart may be due to its ability to reduce parasitemia. Lonart is a combination of 80 mg Artemether and 480 mg Lumefantrine. It acts within the malaria parasite causing morphological changes within the parasite membranes due to the presence of the endoperioxide bridge in artemether that causes release of free radicals; while lumefantrine interferes in the hemoglobin polymerization processes. Both compounds also cause morphological changes in ribosomes as well as in the endoplasmic reticulum resulting in inhibition of protein synthesis [37]. Thus, as parasite level decreased, ACE and Ang II also decreased, validating the impact of RAS in the pathogenesis of malaria [16,20]. In fact, some studies have suggested changes in structure of RAS peptides, in order to obstruct their antihypertensive effects without damaging their anti-plasmodial effects [14,17,18,19]. This has led to the synthesis of angiotensin-restricted analogs with anti-plasmodial effects on *P. falciparum* and *P. gallinaceum* [13,17,18,19,37].

In this study, the significantly ($P<0.05$) high serum and kidney activity of ACE and levels of Ang II observed in infected mice, was found to have occurred at gene expression level, as ACE and AT_1R genes were significantly ($P<0.05$) expressed. The increased mRNA expression of ACE and AT_1R indicate that RAS is essential in malaria infection and, may be targeted to prevent malaria [16,21,25]. This agrees with studies demonstrating that AT_1R expression is upregulated during blood-stage *P. berghei* infection [7,12], resulting in increased integrin and chemokine receptors expression, as well as higher cytokine production. This in turn, stimulates $CD8^+$ T cell migration and segregation in the brain to stimulate edema and cause the life-threatening situation observed in CM [20]. T cells are capable of inducing autocrine and/ or paracrine cellular effects by converting angiotensinogen to active Ang II; while Ang II mediates the activation, migration and adhesion of T-cells via AT_1R in mice infected with *P. berghei* ANKA strain [7]. These therefore, imply that inhibition of this pathway may be beneficial for survival of infected mice.

The down regulation of ACE and AT_1R mRNA in mice treated with 0.03 mg/kg lonart and captopril at 0.03 and 0.09 mg/kg (Figs. 2 and 3), clearly indicates that captopril, effectively suppresses malaria parasite by decreasing the concentrations of ACE and Ang II; and also decreasing renal expression of ACE and AT_1R mRNA. These results are consistent with reports demonstrating that Ang II/AT1 receptor inhibition with captopril or losartan decreased parasitemia and improved well-being of infected mice [7,16,19]. These data suggest that antagonists of AT_1R and ACE inhibitors may increase the resistance of mice against CM, thus increasing survival rate.

5. CONCLUSION

Captopril was shown to affect Ang II and ACE concentration, as well as the expression of ACE and AT_1R. It can be concluded from this study that the captopril decreased parasitemia by decreasing levels of Ang II and ACE, and this may be at the level of gene expression, due to the down-regulation of ACE and AT_1R in kidneys of malaria-infected mice. Thus, ACE inhibitors like captopril may be valuable against malaria, and may possess the potential therapeutic effect as adjunctive treatment for malaria, although further studies are required to establish this.

ETHICAL APPROVAL

As per international standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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