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The Role of Metfirnin and Artesunate-amodiaquine Combination in Preventing Cerebral Malaria in Mice

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ABSTRACT

Background and objective: Cerebral malaria is one of the important complications of malaria infection responsible for most malaria-related cases of death. This study investigated the antiplasmodial and some neurological effect of metformin prophylaxis and artesunate-amodiaquine curative regimen in *Plasmodium berghei* infected male mice.

Methods: Sixty male mice were randomly divided into six groups labeled I to VI with each group containing ten mice. Group I was normal control (NC) that received water and feed *ad libitum*, group II was negative control parasitized with *P. berghei berghei* and not treated, while group III wast positive control parasitized and treated with artesunate-amodiaquine. Group IV received metformin prophylaxis for seven days before pasitemia induction and not treated thereafter. Group V received metformin for seven days, parasitized and later treated with artesunate-amodiaquine. Group VI were treated with metformin and artesunate-amodiaquine combination after parasitemia induction. Initial parasitemia was established after 72 hours using tail-blood film microscopy, and final parasitemia was determined 24 hours after the last treatment. All post-infection treatments lasted for three days, after which the animals were euthanized. The hippocampal tissues were harvested, and some homogenized and the homogenate used for biochemical assay; while some others were used histological examination.

Results: Parasitemia level was significantly ($p < 0.05$) decreased in treated groups, especially in the group that received both metformin prophylaxis and artesunate-amodiaquine post-infection treatment (group V). The oxidative stress biomarkers, inflammatory biomarkers and neurotransmitters in the hippocampal homogenates of treated groups especially group V were significantly ($p < 0.05$) restored close to those of normal mice.

Conclusions: This study concludes that metformin could be repurposed as a good prophylactic agent to artemisinin-based combination therapies against Plasmodium malaria infection. This implies that metformin prophylaxis might help in combating Plasmodial malaria resistance; thus, prevent complications such as cerebral malaria.

Keywords: Metformin, Artesunate-amodiaquine, *P.berghei*, Cerebral malaria, Neurotransmitters

INTRODUCTION

Malaria is one of the global most critical public health concerns, particularly in Sub-Saharan Africa and Southeast Asia. The disease is caused by Plasmodium parasites borne by female *Anopheles* mosquitoes which introduce them into the host during blood meal [1]. In humans, the parasites find their way to the liver which is

their maturity domain and then back to the bloodstream where they attack the red blood cells as source of nutrients [2]. The species of Plasmodium parasites that cause malaria infection in human are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*; with *P. falciparium* being responsible for over 99 % of malaria in Africa [3]. The general symptoms of malaria include fever**, s**weating, headache, fatigue, malaise, muscle aches, body pains, nausea and vomiting, diarrhea**,** loss of appetite, abdominal pain and cough; however, the most common symptoms are fever and chills [1]. Malaria causing species in rodents are different and include *Plasmodium berghei berghei*, *Plasmodium knowlesi*, and *Plasmodium cynomolgi* [4].

The most important complications of severe malaria responsible for malaria-related death cases among children and pregnant women in endemic regions are cerebral malaria (CM), severe anaemia, respiratory distress (acidosis) and hypoglycemia which can manifest at the same time or individually. Severe malaria results from long time exposure to the infection causing oxidative stress burst due to Plasmodium parasites attacking the red blood cells. Infected erythrocytes through specific ligands interact with and are sequestered in endothelial cells thereby causing dysfunctions of organs such as the brain and kidney [5].

Cerebral malaria result from microvascular sequestration of infectected red blood cells in the brain leading to increased compromise of the blood brain barrier integrity through endothelial activation, tight junction protein loss, resetting and inlammation [6]. Manifestations of this complication cause a progressive loss of cognitive abilities, seizures, coma and eventually, death [7]. It is very lethal and causes death of children under five years of old [8]. Survivors of the disease often develop neurologic deficits sometimes after 2 years of post-infection and treatments [9]. It was reported that the use of antimalarials alone could not curb the devastating manifestations of cerebral malaria. Though treatment with artesunate was said to improve survival, mortality remained high, and so, adjunctive therapies are still urgently needed. However, the use of antioxidants in reducing the risk and severity of cerebral malaria has been noted since most of the antimalarials act as pro-oxidants, increasing the oxidative stress together with the Plasmodium [10].

Metformin, traditionally used as an oral hypoglycemic agent for treatment of type 2 diabetes, has been repurposed for the prevention of malaria [11]. *In vitro* and *in vivo* study revealed that metformin impaired development of *P. berghei* liver-stage in mice, significantly reducing liver-schizont size and infected hepatocyte numbers [11]. *Plasmodium* infection induces lipogenic state which is reversed via adenosine monophosphate kinase (AMPK) activation following metformin pretreatment. This limited lipid availability to the parasites inhibites the intraerythrocytic growth of the parasites [12], and thus may prevent development of complications. Furthermore, metformin was suggested to show synergistic effects with antimalarials like atovaquone, reducing *P. falciparum* prevalence more effectively than monotherapy [12]. This study investigated whether metformin and artesunate-amodiaquine combination could prevent Plasmodium parasite-induced neurodysfunction.

METHODOLOGY

Experimental Animals

Sixty (60) male mice of eight weeks old were used in this study. The mice were obtained from the Animal House of the Faculty of Pharmacy, University of Uyo, Uyo, Nigeria. The animals will be acclimatized for seven days in a ventilated room in wooden cages with wire mesh on top at the Animal House of the Faculty of Pharmacy, University of Uyo. They were maintained under standard conditions of humidity (50 ± 5 %) and temperature (28 ± 32 ^{O}C), and exposed to a 12-hour light-dark cycle. They were given livestock feed (vital grower) and water *ad libitum*. The experiment was done in accordance with the ethical approval by The Faculty of Basic Medical Sciences Research and Ethical Committee, University of Uyo.

Experimental Design and Treatment of Animals

The design of the experiment is as shown in Table 1. After the seven days of acclimatization, the 60 mice were randomly divided into six groups labeled “I” to “VI” with each group containing 15 mice. Group I was the normal control group that received distilled water *ad libitum*. Group II animals served as negative controls to

be infected with *Plasmodium berghei* and not treated. Group III was infected with *P. berghei* and treated with artesunate-amodiaquine (AA) at the dose of 11.43 mg/kg body weight (bw). Group IV animals received metformin prophylaxis at the dose of 14.29 mg/kg bw for seven days before infecting them with *P. berghei berghei*, and not treated thereafter. Group V animals received metformin prophylaxis at the dose of 14.29 mg/kg bw for seven days, infected with *P. berghei* and later treated with AA at the dose of 11.43 mg/kg bw. Animals in group VI were infected with *P. berghei* and then treated with metformin (14.29 mg/kg bw) and artesunate-amodiaquine (11.43 mg/kg bw) concomitantly. In all infected mice, parasitemia was established after a 72-hour window period via tail-blood film microscopy before commencement of a three-day treatment regimen. The whole experiment is designed to last for 13 days.

Table 1: Experimental Design

Grouping/Treatment	No. of Mice	Administration Period in Days before P Infection	Treatment Period in Days after P Infection
I (Normal control (distilled water))	10	-	-
II (Negative control: P + No treatment)	10	-	-
III (P+AA (11.43 mg/kg bw))	10		3
IV (Met (14.29 mg/kg bw) + P + No treatment)	10	7	-
V (Met (14.29 mg/kg bw) +P + AA (11.43 mg/kg bw))	10	7	3
VI (P + AA (11.43 mg/kg bw) + Met (14.29 mg/kg bw))	10	-	3

Parasite Inoculation

Following the experimental design, parasite inoculation was done using the method described by [13] with little modifications. The parasite was obtained from a donor mouse at the Animal House of the Faculty of Pharmacy, University of Uyo by cardiac puncture, after anaesthetizing with ketamine. The parasite-infected blood was diluted with normal saline and 0.4 mL of the infected blood containing about 1×10^7 parasitized erythrocytes was passaged intraperitoneally into each of the mouse according to the study design. Parasitemia was confirmed in the animals after 72 hours of parasite inoculation through microscopic examination of stained blood films from the tail of the mice. Final parasitemia was determined 24 hours after the last post-infection treatment using tail blood film microscopy after staining.

The viewing and counting of parasitized erythrocytes was done using the low-power (10×) objective of a microscope. The percentage parasitemia was determined by counting the number of parasitized erythrocyte and calculated according to the following formula adopted by [14]:

$$\% \text{ Parasitemia} = \frac{\text{Total number of parasitized RBCs}}{\text{Total number of RBCs}} \times 1000$$

Animal Sacrifice and Collection of Samples

After the last post-infection treatment, the animals were fasted overnight but with free access to water. They were euthanized with ketamine (40 mg/kg) anesthesia (Sigma Aldrich, Germany). The hippocampal tissues were harvested from seven mice in each group. A team of 3 persons was used to ensure speedy process. All equipment and solutions were prepared in advance and kept chilled on ice. The hippocampal tissues were quickly harvested while the hearts still beat and immediately transferred to pre-chilled plain tubes in ice bath, and later homogenized. Some of the tissues were used for histological staining.

Antioxidant Determination

Superoxide dismutase **(**SOD) activity was determined spectrophotometrically as described by Marklund and Marklund [15]. Catalase (CAT) activity wwas assayed by measuring the degradation rate of H_2O_2 using the method by Goth {16]. The Flohé and Gunzler [17] method was used for the glutathione (GSH) and glutathione peroxidase (GPx) activity determination. The assay of lipid peroxidation using malondialdehyde (MDA) was determined by the method described by Reilly and Aust [18].

Determination of Inflammatory Biomarkers

Interleukin-1b (IL-1b), tumor necrotic factor alpha (TNF-*a*), interleukin-4 (IL-4) and interleukin-10 (IL-10) were assayed for using automated clinical immunoassay analyzer (chemiluminescent assays) as described by Israeli *et al*. [19]. The concentration of nitric oxide was measured using Griess (diazotization) colorimetric method as described by Brizzolari *et al*. [20].

Neurotransmitters Determination

Neurological examinations will be carried out to ascertain the concentrations of some neurotransmitters such as acetylcholine, glutamate, gamma aminobutyric acid and serotonin using mass spectrometry method as described by Panteghini and Bonora [21].

Histological Assessment

Some of the harvested hippocampal tissues from each group were fixed in 10 % phosphate-buffered formaldehyde. Following standard procedures [22], tissues were dehydrated, cleared, embedded in wax, sectioned on a rotary microtome, mounted on clean glass slides, stained and counter stained with Haematoxylin and Eosin respectively [23]. Photomicrographs were generated through a digital camera AmScope® mounted on a light microscope (Olympus CX31).

Statistical Analysis

The data was subjected to analysis using the Statistical Package for Social Sciences (SPSS), version 25.0. Data obtained was expressed as mean ± standard error of mean (SEM). Multiple group comparisons were based on ONE-Way Analysis of variance (ANOVA) followed by least significant difference (LSD) multiple comparison (post hoc test). Values of $p < 0.05$ were considered significant.

RESULTS

Antiplasmodial Activity of Metformin and Artesunate-amodiaquine Combination Therapy in Mice

The parasitemia count significantly ($p < 0.05$) decreased in all test groups when compared with negative control group II which were parasitized and not treated. The parasetemia reduction was more significant ($p < 0.05$) in group V which received metformin before parasitimia induction and later treated with artesunate-amodiaquine (Table 1).

Oxidative Stress Biomarkers in Hippocampus Homogenate

Lipid peroxidation as asay vis-à-vis MDA significantly ($p<0.05$) decreased in all test groups (III - VI) when compared with group II. The antioxidant systems significantly ($p<0.05$) improved in all the test group, more prominently in group V, when compared with group I (Table 2).

Inflammatory Biomarkers in Hippocampus Homogenate

There were significant ($p<0.05$) improvements in the concentrations of nitric oxide, IL-1b, TNF-*a*, IL-10 and IL-4 in all the test groups when compared with group I. The improvement was more pronounced in group V (Table 3).

3.4 Concentrations of some Neurotransmitters

The significantly ($p<0.05$) altered neurotransmitters in group II when compared with group I showed significant restoration in all the test groups, especially in group V (Table 4).

Table 1: Antiplasmodial Activity of Concomitant Administration of Artesunate amodiaquine Metformin in Male Mice

Groups / Treatments	Before Treatment	After Treatment	Change in parasite count	% change in parasite count
I (Normal control: 1 ml distilled water)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
II (Negative control: P)	55.51 ± 0.56	56.34 ± 0.44	0.83 ± 0.64	1.53 ± 1.15
III (Positive control (P+AA (11.43 mg/kg bw))	54.28 ± 0.56	8.48 ± 0.12	-45.80 ± 0.61	-84.37 ± 0.32^{b}
IV (Met (14.29 mg/kg bw) +P (11.43 mg/kg bw))	36.90 ± 0.33	36.93 ± 0.23	0.03 ± 0.12	0.09 ± 0.32^{bc}
V (Met (14.29 mg/kg bw)+P+AA (11.43 mg/kg bw))	35.71 ± 0.15	3.91 ± 0.12	-31.80 ± 0.18	-89.05 ± 0.33^{bcd}
VI (P+AA (11.43	54.57 ± 0.55	7.96 ± 0.09	-46.61 ± 0.52	-85.41 ± 0.18^{bde}

Results presented as Mean ± Standard Error of mean (SEM), n = 15; $p < 0.05$ considered as significant.

a = significantly different when all test groups are compared with normalcontrol group I

b = significantly different when groups III, IV, V and VI are compared with negative control group II

c = significantly different when groups IV, V and VI are compared with positive control group III

d = significantly different when groups V and VI are compared with group IV

e = significantly different when group VI is compared with group V

P = *Plamodium berghei berghei*

AA = Artesunate-amodiaquine

Met = Metformin

Bw = body weight

Table 2: Hippocampal Oxidative Stress Biomarkers in *Plasmodium berghei* Infected Mice Treated with Metformin and Artesunate-amodiaquine Combination

Groupings/ treatment	Brain MDA (µM)	Brain SOD (U/ml)	Brain Catalase (U/mg prot.)	Brain GPx (U/L)	Brain GSH (mM)
I (Normal control: 1 ml distilled water)	3.79±0.11	1.33±0.01	57.59±0.67	54.04±1.19	0.17±0.01
II (Negative control: P)	10.26±0.18^{a}	0.13±0.05^{a}	27.22±1.27^{a}	25.67±0.85^{a}	0.10±0.01^{a}
III (Positive control (P + AA (11.43 mg/kg bw))	6.05±0.26^{ab}	0.97±0.0^{ab}	50.94 0.57^{ab}	47.89±1.86^{ab}	0.15±0.0^{ab}
IV (Met (14,29 mg/kg bw) +P)	8.47±0.32^{abc}	0.35±0.01^{abc}	35.89±0.49^{abc}	32.18±0.92^{abc}	0.13±0.0^{abc}
V (Met (14,29 mg/kg bw) + P + AA (11.43 mg/kg bw))	3.90±0.07^{bcd}	1.29±0.0^{bcd}	56.84±0.88^{bcd}	53.79±1.26^{bcd}	0.17±0.01^{bcd}
VI (P+AA (11.43 mg/kg bw) +Met (14,29 mg/kg bw))	6.03±0.06^{abde}	0.97±0.02^{abde}	50.87 ± 0.72^{abde}	48.46±0.83^{abde}	0.15 ± 0.0^{abde}

Results presented as Mean ± Standard Error of mean (SEM), n = 15; $p < 0.05$ considered as significant.

a = significantly different when all test groups are compared with normalcontrol group I

b = significantly different when groups III, IV, V and VI are compared with negative control group II

c = significantly different when groups IV, V and VI are compared with positive control group III

d = significantly different when groups V and VI are compared with group IV

e = significantly different when group VI is compared with group V

P = *Plamodium berghei berghei*

AA = Artesunate-amodiaquine

Met = Metformin

bw = Body weight

Table3: Hippocampal Homogenate Inflammatory Biomarkers in *Plasmodium berghei* Infected Mice Treated with Metformin and Artesunate-amodiaquine Combination

Groupings/ treatment	Brain NO (µM)	Brain IL-1B (pg/ml)	Brain TNF-A (pg/ml)	Brain IL-10 (pg/ml)	Brain IL-4 (pg/ml)
I (Normal control: 1 ml distilled water)	3.94±0.07	88.86±0.96	34.0±0.66	26.56±0.76	94.60±1.50

II (Negative control: P)	11.26±0.17^{a}	131.35±1.76^{a}	88.61±2.27^{a}	43.57±0.51^{a}	33.68±1.57^{a}
III (Positive control (P + AA (11.43 mg/kg bw))	5.31±0.08^{ab}	100.01±1.86^{ab}	46.01±1.27^{b}	33.74±0.94^{b}	82.48±3.20^{b}
IV (Met (14.29 mg/kg bw) +P)	8.01±0.12^{abc}	108.87±0.83^{abc}	73.11±2.60^{abc}	38.67±0.76^{abc}	52.78±1.36^{abc}
V (Met (Met (14.29 mg/kg bw) + P + AA (11.43 mg/kg bw))	4.03±0.23^{bcd}	89.21±1.20^{bcd}	35.20±2.57^{bcd}	27.57±0.86^{bcd}	93.77±1.23^{bd}
VI (P+AA (11.43 mg/kg bw) + Met (14.29 mg/kg bw))	5.28±0.14^{abde}	100.03±3.48^{abde}	46.04±1.06^{abde}	33.86±0.74^{abde}	81.36±1.5^{abde}

Results presented as Mean ± Standard Error of Mean (SEM), n = 15; $p < 0.05$ considered as significant.

a = significantly different when all test groups are compared with normalcontrol group I

b = significantly different when groups III, IV, V and VI are compared with negative control group II

c = significantly different when groups IV, V and VI are compared with positive control group III

d = significantly different when groups V and VI are compared with group IV

e = significantly different when group VI is compared with group V

P = *Plamodium berghei berghei*

AA = Artesunate-amodiaquine

Met = Metformin

bw = Body weight

Table 4: Some Hippocampal Homogenate Neurotransmitters in *Plasmodium berghei* Infected Mice Treated with Metformin and Artesunate-amodiaquine Combination

Groupings/ treatment	**Brain ACh (U/ml)**	**Brain Glutamate (mM)**	**Brain GABA (pg/ml)**	**Brain Serotonin (pg/ml)**
I (Normal control: 1 ml distlled water)	0.18±0.01	245.15±3.01	281.38±2.70	154.7 1.57
II (Negative control: P)	0.04±0.01^{a}	300.02±1.26^{a}	351.77±2.54^{a}	209.42±0.82^{a}
III (Positive control (P + AA (11.43 mg/kg bw))	0.13±0.01^{ab}	261.47±1.05^{b}	304.97±2.06^{ab}	174.03±5.76^{ab}
IV (Met (14.29 mg/kg bw) +P)	0.09±0.00^{abc}	274.50±1.85^{ab}	334.05±1.58^{abc}	192.45±1.66^{abc}
V (Met (14.29 mg/kg bw) +P + AA (11.43 mg/kg bw))	0.16±0.00^{abcd}	249.59±2.25^{b}	284.72±2.26^{bcd}	159.84±3.41^{bcd}
VI (P+AA (11.43 mg/kg bw) +	0.12±0.0^{abde}	260.46±2.24^{b}	305.03±1.48^{abde}	174.93±5.09^{abde}

Met (14.29 mg/kg bw))				

Results presented as Mean ± Standard Error of Mean (SEM), n = 15; $p < 0.05$ considered as significant.

a = significantly different when all test groups are compared with normalcontrol group I

b = significantly different when groups III, IV, V and VI are compared with negative control group II

c = significantly different when groups IV, V and VI are compared with positive control group III

d = significantly different when groups V and VI are compared with group IV

e = significantly different when group VI is compared with group V

P = *Plamodium berghei berghei*

AA = Artesunate-amodiaquine

Met = Metformin

bw = Body weight

Hippocampal Tissue Histology

The treatments ameliorated hippocampal malaria-related damage mostly in group V as shown in fIg, 1.

Figure 1: Hippocampal tissue photomicrographs of normal mouse (4.1a), parasitemia induction (4.1b), parasitemia induction + AA (11.43 mg/kg bw) (4.1c), metformin (14.29 mg/kg Bw) prophylaxis + parasitemia induction (4.1d), metformin prophylaxis + parasitemia induction + AA (4.1e), and parasitemia induction + metformin + AA (4.1f) (Mag. x400).

(4.4a): Positive control group I – Normal hippocampus architecture. No pathology.

(4.4b): Negative control group II – Hippocampal tissue showing average sized pyramidal layer across the Dentate gyrus (DG), hyperplastic neurons at the CornuAmmonis (CAs) region and hypotrophic neuron.

(4.4c): Group III - Hippocampal tissue showing average sized pyramidal layer across the CAs regions and DG, and focal shrunkened neurons in the DG.

(4.4d): Group IV - Hippocampal tissue showing average sized pyramidal layer across the CAs regions and DG, mild shrunken neurone with pyknotic nucleus in the CA3 region of the CAs.

(4.4e): Group V - Hippocampal tissue showing an average-sized pyramidal layer across the DG and few hypotrophic neurons and hyperchromatic nuclei.

(4.4f): Group F - Hippocampal tissue showing average sized pyramidal layer across the CAs regions and DG, and sporadic shrunken neurone in the CA4 region of the CAs.

AA – Artesunate-amodiaquine

DISCUSSION

The preventive and curative models adopted in this present study revealed that metformin and artesunate-amodiaquine combination significantly ($p<0.05$) reduced parasitemia in all the treated groups (III-VI). The parasite clearance was more significantly ($p<0.05$) prominent in group V which received metformin before plasmodia induction and later treated with artesunate-amodiaquine compared with group III that was

parasitized and treated with artesunate-amodiaquine. The group III had percentage parasitemia change of -84.37 ± 0.32 % which was significantly ($p<0.05$) higher than that recorded for group V (-89.05 ± 0.33 %). The results correlate with the report of Vera *et al* [11] that the chemopreventive potential of metformin against malaria infection could be enhanced by combination with conventional antimalarials. Similarly, Mokogwu *et al*. [24] reported that prophylactic use of methanol extract of *Salvia officinalis* resulted in a significant dose-dependent reduction of parasitemia in *P. berghei* infected mice. Also, Udeme *et al*. [25] noted in their study that prophylactic use of artemether-lumefantrine-tinidazole significantly reduced parasite load in *P. berghei* infected mice.

The assessment of oxidative stress biomarkers in hippocampal tissue homogenate showed significantly increased MDA concentration with significantly decreased SOD, CAT, GPx and GSH concentrations in group II compared with group I. These were indicatives of oxidative stress and neuro-inflammation which could likely cause synaptic dysfunction, impaired neurogenesis and memory/cognitive deficits associated with cerebral malaria infection [26]. The increased concentration of MDA in this study corroborates the finding of Raza *et al*. [27] where MDA level increased proportionally with parasitemia in cerebral malaria. Prabhu *et al*. [28], showed negative correlation between GPx concentrations and MDA concentrations in *P. vivax* and *P. falciparum* infected patients; while Opajobi *et al*. [29] reported same in mice. The results of antioxidant systems recorded here corroborate the findings of Adegoke *et al*. [30] who reported reductions in CAT, GPx, and GSH concentrations in the brain of *P. bergei* infected mice. Opajobi *et al*. [29] recorded reduced activities of CAT and SOD in the brain of *Plasmodium berghei* infected mice.

All the treated groups showed significantly reduced MDA concentration, and significantly elevated SOD, CAT, GPx and GSH concentrations compared with group II. These changes were significantly more pronounced in group V. It indicated that the treatment regimens halted parasite-initiated oxidative stress and inflammatory injuries and boosted antioxidant systems. This agrees with the report of Adegoke *et al*. [30] that prophylactic administration of ethanol leaf extract of *Musa paradisiaca* significantly reduced oxidative stress and improved concentrations of antioxidant systems in the brain of *P. berghei* infected mice when compared with mice not admistered prophylactic treatment of the extract.

The inflammatory biomarkers assayed in hippocampal homogenate were nitric oxide (NO), IL-1β, TNF-*a*, IL-10 and IL-4. Nitric oxide regulates vasodilation, neurotransmission, and immune responses in many physiological systems such as the cardiovascular, nervous, and immune systems [31]; serving also as a potent antimicrobial agent within the host's innate immune system [32]. Upon infection, inducible synthase is upregulated in immune cells, leading to increased production [33] with subsequent direct inhibition of replication or killing of the pathogens [34].

This study showed significantly elevated NO, IL-1β, TNF-*a* and IL-10 concentrations, and significantly reduced IL-4 concentration in group II compared with group I. This trend of changes signified neuro-inflammatory dysregulation. High concentration of NO is implicated in neuronal apoptosis and cognitive impairment in cerebral malaria while low concentration is vital for vasodilation and blood–brain barrier integrity. The increase in NO concentration agrees with the report of Silva *et al*. [35] where elevated expression of inducible nitric oxide synthase (iNOS) was associated with cognitive impairment (memory and learning deficits) and nitrosative stress (peroxynitrite formation) in a mouse model of cerebral malaria. On the other hand, Vathsala *et al*. [36] in their study showed that increased production of NO was an important factor in eliminating malaria parasite. Barbosa *et al*. [37] confirmed increased mortality in *Plasmodium berghei* infected mice after the inhibition of NO synthesis. Similarly, inhaled nitric oxide was said to have neuroprotective effect among severe malaria children [38]. The elevated concentrations of TNF-*a* and IL-1β recorded in this study agree with Freire-Antunes *et al.* [39] who reported increase in concentrations of pro-inflammatory cytokins and NO in experimental cerebral malaria model. Also, Schiess *et al*. [40] recorded significant increase in TNF-*a* and IL-1β concentrations in cerebral malaria compared with healthy control.

The increase in IL-10 is compensatory, helping to suppress the elevated TNF-α, IL-1β, and iNOS concentrations. The upregulation was induced by the host's immune system to counter the effect of increased pro-inflammatory cytokines. This agrees with Tembo *et al*. [41] who reported elevated IL-10 in children with

cerebral malaria. Kumar *et al*. [42] noted in their study that increased IL-10 concentration was compensatory, helping to suppress elevated pro-inflammatory cytokines. The reduced concentration of IL-4 indicated less down-regulation of inflammation; thus, contributing to neuro-inflammation, endothelial activation, blood–brain barrier damage, and neuropathology. This correlates with the report of Wu *et al*. [43] where low serum contration of IL-4 was recorded in cerebral malaria.

In all the treated groups, concentrations of NO, TNF-*a*, IL-1β and IL-10 significantly reduced with significantly increased IL-4 concentration compared with group II. This effect is more significantly prominent in group V. This implied notable parasite clearance, reversal of systemic and neuro-inflammatory damage, and normalization of concentrations of the cytokines. These are are in line with the work of Mukherjee *et al*. [44] where there were decreases in the secretions of pro-inflammatory cytokins (TNF-*a* and IL-1β) and increase in anti-inflammatory cytokine (IL 4) in the brain of *P.berghei*-infected mice ttreated with artemisinin-chloroquine combination therapy compared with untreated mice. Similar observations were made by Guiguemde *et al*. [10] in the treatment of murine cerebral malaria with dihydroartemisinin and artesunate when compared with untrerated *P. berghei* infected mice.

Neurotransmitters are critical components of central and peripheral nervous systems which serve as signaling molecules, enabling nerve cells to efficiently convey information both electrically and chemically [45]. They can be excitatory (examples acetylcholine and glutamate) or inhibitory (examples gamma-aminobutyric acid and serotonin) neurotransmitters [46]. Neurological impairments, including cognitive, behavioral, and motor dysfunctions have been reported to be associated with cerebral malaria due to disturbances in neurotransmitter balances [47].

The present study showed significantly reduced acetylcholine (ACh), and significantly elevated glutamate (Glu), gamma-aminobutyric acid (GABA) and serotonin (5-HT) in group II compared with group I. This pattern of changes reflected cholinergic hypofunction, glutamatergic excitotoxicity, inhibitory overcompensation and neuroimmune serotonin dysregulation which promote synaptic dysfunction, neuronal apoptosis, and cognitive deficits characteristic of cerebral malaria neuropathology [48]. The reduced ACh concentration could be due to increased concentrations of glutamate and GABA as recorded by Malomouzh *et al*. [49].

These results correlate with the report of Oliveira *et al*. [50] who recorded decreased ACh concentration, and increased glutamate, GABA and 5-HT concentrations in mice with cerebral malaria. Also, Daniyan *et al*. [51] in their study noted increased glutamate concentration in cerebral malaria while Lopes de Souza *et al*. [52] observed increased GABA concentration in mice with cerebral malaria than in uncomplicated malaria cases. However, the elevated serotonin concentration recorded in this study disagrees with Briggs *et al*. [53] and Das *et al*. [54] who reported reduced serotonin level in mice with cerebral malaria compared with healthy control.

In all the treated groups, especially group V, the concentration of ACh significantly increased with significantly decreased glutamate, GABA and 5-HT concentrations compared with group II. This implied normalization of the neurotransmitters due to reversal of neuro-inflammation and cytokine pool, and improved antioxidants status. The results agree with Sanguanwong *et al*. [55] who reported that combined astragaloside-iv and artesunate treatment showed neuroprotective and anti-neuroinflammatory effect in *Plasmodium yoelii* infection; although they did not quantify the neurotransmitters in their experiment. The decrease in serotonin concentration recorded in all the treated groups agrees with the work of Islahudin *et al*. [56] where quinine interfered with serotonin biosynthesis and action. Similarly, Thompson and Lummis [57] noted that antimalarial drugs inhibited $GABA_A$ and serotonin receptors. However, there is dearth of literature that quantify the concentrations of neurotransmitters during malaria infection and after malaria treatment in both human subjects and experimental models.

The effect of metformin and artesunate-amodiaquine combination on the morphology of hippocampal tissues in *Plasmodium berghei berghei* infected mice is shown in Figure 1. Average sized pyramidal layer across the Dentate gyrus but hyperplastic neurons at the Cornu Ammonis region suggesting an adaptive response to an injury or stress were observed in Fig. 1b. The neurons were reduced in size (hypotrophy) and depicted

hyperchromatic (densely packed DNA or chromatin) nucleus. This corroborates the reports of Alharbi *et al*. [58] where *P. berghei* infection caused some histopathological alterations in the cerebellum of infected mice. Fig. 1c revealed average sized pyramidal layer across the CornuAmmonis regions and Dentate gyrus. The neurone depicted central large vesicular nuclei, containing at least one nucleolus. However, there was a focal shrunken neuron in the inner polymorphic area of the dentate gyrus. Fig. 1d had average sized pyramidal layer across the CornuAmmonis regions and Dentate gyrus. There were mild shrunken neurons with pyknotic nucleus in the region of the cornuAmmonis depicting decreased damage in the brain architectures due to mediating role of metformin against Plasmodium parasite-related assaults on the brain tissue. In Fig. 1e, normal average-sized pyramidal layer across the Dentate Gyrus was observed. This could indicate protection which may be due to prophylactic use of metformin. Fig. 1f showed average sized pyramidal layer and Dentate gyrus, with mild sporadic shrunken neurons having pyknotic nucleus in the region of the CornuAmmonis.

There is dearth of direct information on effect of prophylactic treatment before malaria infection and subsequent treatment with standard antimalarial therapy on hippocampus morphology. However, it might be inferred that if prophylaxis effectively lowered parasite burden or delayed parasitaemia, hippocampal lesions would be reduced, and neuronal integrity better preserved compared with infected untreated animals. Upon subsequent standard drug treatment, further mitigation of damage would occur. Nonetheless, Alharbi *et al*. [58] reported that malaria treatment using *Juglans regia* improved *P. berghei*-related brain damage in mice. Also, Sharma *et al*. [59[and Rusu *et al*. [60] reported that flavonoids in *Juglans regia* could protect brain cells by acting as free radical scavengers, inhibiting DNA damage and mutagenicity.

CONCLUSION

The evaluation of the role of metfirnin and artesunate-amodiaquine combination in preventing cerebral malaria in mice revealed that taking metformin prophylaxis before exposure to Plasmodium malaria infection can significantly inhibit parasite replication, and subsequent administration of artesunate-amodiaquine maximally lowers parasite load in mice. This treatment regimen was observed to ameliorate malaria-related neuronal disturbances, and minimized parasite-associated oxidative stress and inflammatory damage on the hippocampal tissue.

COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

Authors declare no conflicts of interest.

Statement of ethical approval

The authors declare compliance with the “Principles of Laboratory Animal Care” (NIH publication No. 85-23, revised 1985) and relevant national laws. All experiments received the University of Uyo’s ethics committee approval.

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