Original Research Article

Safety evaluation of artemether-lumefantrine-tinidazole on the kidneys of healthy and diseased mice

Running title: Safety of artemether-lumefantrine-tinidazole on the kidneys of mice

ABSTRACT

Tinidazole-artemether-lumefantrine (A/L/T) can be use for the treatment of malaria; therefore its safety assessment is imperative. This study assessed the safety of A/L/T on the kidney of healthy and diseased mice. Sixty mice were randomized and used for this study. Mice were diseased with *Plasmodium berghei* (1×10^7) and treated with T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T for 4 days. Healthy mice were treated with T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T for 28 days. At the termination of treatment, the mice were weighed, sacrificed and kidneys were collected and weighed. Blood samples were collected and examined for liver function markers. Kidneys were evaluated for oxidative stress markers and histology. All evaluated parameters were normal in diseased mice treated with A/L/T when compared to control. Body weight was decreased ($p<0.01$) whereas kidney weight ($p<0.01$) was increased in healthy mice treated with A/L/T when compared to control. Healthy mice treated with A/L/T showed impaired renal function marked by significantly ($p<0.001$) elevated levels of serum creatinine, urea, uric acid with significantly ($p<0.001$) decreased levels of albumin, and total protein when compared to control. In healthy mice treated with A/L/T, altered kidney oxidative stress markers were characterized by significantly ($p<0.001$) decreased glutathione, catalase, glutathione peroxidase, superoxide dismutase levels with significantly ($p<0.001$) increased malondialdehyde levels when compared to control. Kidneys of A/L/T treated healthy mice showed tubular necrosis and enlarged Bowman's space. The use of A/L/T as an antimalaria may be safe on the kidney, but long term use may impair kidney function.

Key words: Artemether-lumefantrine, tinidazole, kidney, toxicity, *Parasite,* mice

1. INTRODUCTION

The kidney is required for several important functions including the sustenance of homeostasis, detoxification, and excretion of toxic drugs and metabolites, thus it is venerable to toxicant induced damage [1]. A number of drugs including amtimalarial drugs (quinine, and artesunate) have been associated with nephrotoxicity [2]. The presentation of nephrotoxicity varies from an acute or chronic decreased glomerular filtration rate to glomerular and tubular damage [3]. Although renal impairment is often reversible if the offending drug is discontinued, the condition can be costly and may require multiple interventions, including hospitalization [4].

Artemisinin based combinations including artemether–lumefantrine (AL), artesunate–amodiaquine, artesunate–mefloquine are the currently recommended antimalarial therapy [5]. Artemisinin derivatives are recommended worldwide for treatment of malaria because of their high potency, rapid onset of action, broad malaria stage specificity, and favorable safety profile [6]. Artemether-lumefantrine has shown efficacy against uncomplicated P. falciparum malaria and chloroquine resistant P. falciparum [7]. It accounted for 73 % of ACTs procured in 2013 [8]. However, artemether-lumefantrine may have potential nephrotoxic effect. Reversible nephrotoxicity, diminished glomerular filtration rate and increased urinary excretion of electrolytes have been reported [9, 10].

Tinidazole (5-nitroimidazole drug) (T) is which has proven to be relatively safe is widely used for the treatment of amoebiasis and giardiasis [11]. However, potential antimalaria activity of T has been reported by some scholars. Studies in a chick model parasitized with *Plasmodium gallinaceum* treated with T showed increased survival time [11]. It cures liver stage of relapsing strain of *P. cynomolgi*, in 'Rhesus' macaques (*Macaca mulatta*) and blood stage infection when coupled with chloroquine [12]. Open label human study shows that T monotherapy once followed by weekly doses cleared blood stage infection with no recurrences of *P. vivax* [13]. In previous study, we showed promising antimalarial activity, characterized by prolonged survival and decreased anemia in a mouse model infected with *P. berghei* treated with of tinidazole in combination of artemether-lumefantrine [14]. In-view of the potential of animalarial drug combinations to cause nephroroxicity, this study assessed the safety of artemether-lumefantrine-tinidazole (A/L/T) on the kidneys of healthy and diseased mice.

2. MATERIALS AND METHODS

Adult mice (20-25g) of both sexes were supplied by the animal unit of the Faculty of Basic Clinical Sciences, University of Port Harcourt, Rivers State. The mice were grouped and kept under natural conditions with free access to diet and water. The mice were acclimated for 2 weeks prior to the study

2.1 Parasite inoculation and treatment

Chloroquine sensitive strain of *P. berghei* in donor mice was provided by the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria. Thirty six mice grouped into 5 (I-V) of n=6, groups II-V were parasitized i.p with *P. berghei* containing 1×10^7 parasitized erythrocytes and allowed for 3 days. On day 4, the mice were treated as follows: Group 1: (Normal control) and group II (Parasitized control) were orally treated with normal saline (0.2mL). Groups III-V were treated daily with T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T respectively for 4 days.

2.2 Treatment of healthy mice and sacrifice

Twenty four mice were grouped (n=6) and administered with drugs as follows: Group 1: (Control) was orally administered with normal saline (0.2mL) for 30 days. Groups II-IV were orally administered with T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T for 28 days respectively. After, treatment, the mice were weighed; fasted over night and anesthetized (diethylether). Blood samples were obtained from the heart, centrifuged (1200 rmp for 20minutes) and sera extracted and assessed for biochemical markers. Mice were dissected, kidneys collected and rinsed in saline. Rinsed kidneys were homogenized in 0.1 M Tris-HCl solution buffered (pH 7.4) and centrifuged (2000rmp for 20 minutes). The homogenates were decanted and assessed for oxidative stress markers.

2.3 Serum biochemical markers assessments

Sera were estimated for serum uric acid, creatinine, urea, total protein, albumin, sodium, potassium and bicarbonate using an auto analyzer.

2.4. Oxidative stress marker assay

Kidney glutathione (GSH) was assayed according to Sedlak and Lindsay (1968) [15]. Catalase (CAT) was assayed as explained by Aebi, (1984) [16]. Glutathione peroxidase (GPx) was measured according to Rotruck *et al*. (1973) [17]. Superoxide dismutase (SOD) was estimated as reported by Sun and Zigman (1978) [18]. Malondialdehyde (MDA) MDA was measured as described by Buege and Aust (1978) [19].

2.5 Histology of the kidney

Dissected kidney tissues were cut and fixed in Bouin's solution for 24hr. Kidney tissues were dehydrated in alcohol-graded series processed and embedded in paraffin wax. Sections (5µm each) were cut and stained with Haematoxylin and Eosin on slides. The slides were examined under light microscope and relevant sections photographed.

2.6 Statistical analysis

Data are presented as means ± SEM and assessed by one-way analysis of variance (ANOVA) followed by *Tukey's* multiple range test (Graph Pad Prism 5 Software, San Diego, CA USA). Differences are statistically significant at $p < 0.05$; $p < 0.01$ and $p < 0.001$

Table 1: Effects of artemether/lumefantrine/tinidazole on body and kidney weights of healthy and parasitized mice

Treatment	Final body weight (g)		Absolute kidney weight (g)		Relative kidney weight (%)	
	Healthy mice	Parasitized mice	Healthy mice	Parasitized mice	Healthy mice	Parasitized mice
Control	28.7±2.43	27.2±3.77	0.17±0.03	0.16±0.01	0.59±0.02	0.59±0.06
T	23.5±2.90	27.9±2.58	0.20±0.01	0.15±0.04	0.85±0.06*	0.54±0.01
A/L	24.0±2.57	29.6±3.32	0.21±0.05	0.17±0.06	0.87±0.08*	0.57±0.09
A/L/T	20.9±3.56*	29.9±2.11	0.25±0.09*	0.16±0.02	1.12±0.05π	0.54±0.04

T: Tinidazole, A/L: Artemether/lumefantrine, A/L/T: Artemether/lumefantrine/tinidazole Data as mean ± SEM, n=5, * $p<0.05$ in comparison to control (Healthy mice), π $p<0.01$ in comparison to control (Healthy mice), SEM: Standard error of mean.

Table 2: Effect of artemether/lumefantrine/tinidazole serum renal function markers of healthy and parasitized mice

Treatment	Healthy mice			Parasitized mice		
	Creatinine (mg/dL)	Urea(mg/dL)	Uric acid (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)
Control	0.50±0.03	7.27±0.04	1.37±0.13	0.56±0.09	7.41±0.07	1.42±0.24
T	0.77±0.07*	10.9±0.09*	2.89±0.09*	0.54±0.03	7.33±0.01	1.40±0.45
A/L	0.96±0.03	12.8±0.36	3.05±0.06*	0.57±0.07	7.30±0.05	1.38±0.67
A/L/T	1.67±0.08	20.1±2.71	5.76±0.72π	0.53±0.01	7.40±0.08	1.35±0.44

T: Tinidazole, A/L: Artemether/lumefantrine, A/L/T: Artemether/lumefantrine/tinidazole Data as Mean ± SEM, n=5, * p<0.05 in comparison to control (Healthy mice), π p<0.01 in comparison to control (Healthy mice), SEM: Standard error of mean.

Table 3: Effect of artemether/lumefantrine/tinidazole serum electrolytes of healthy and parasitized mice

Treatment	Healthy mice				Parasitized mice			
	K (mmol/L)	Na (mmol/L)	Cl (mmol/L)	HCO3 (mmol/L)	K (mmol/L)	Na (mmol/L)	Cl (mmol/L)	HCO3 (mmol/L)
Control	4.33±0.03	120.2±11.7	133.7±12.6	12.1±1.71	4.12±0.06	110.1±10.6	127.3±9.22	13.1±1.65
T	4.30±0.90	117.0±10.5	130.4±10.7	12.3±01.42	4.10±0.01	109.1±12.1	125.5±10.6	13.6±1.14
A/L	4.27±0.57	115.6±12.3	128.3±10.5	12.7±1.63	4.09±0.19	107.3±10.9	123.4±11.8	13.3±1.90
A/L/T	4.25±0.56	114.9±10.1	126.2±11.9	12.6±1.24	4.06±0.24	105.4±11.4	122.3±12.5	13.7±1.40

T: Tinidazole, A/L: Artemether/lumefantrine, AL/T: Artemether/lumefantrine/tinidazole
Data as Mean ± SEM, n=5, SEM: Standard error of mean.

Table 4: Effect of artemether/lumefantrine/tinidazole on liver oxidative stress markers of healthy mice

Treatment	MDA nmole/mg protein	GSH µmole/mg protein	CAT U/mg protein	SOD U/mg protein	GPx U/mg protein
Control	0.12 ± 0.02	8.4 ± 0.73	25.6 ± 2.01	14.8± 2.51	15.3 ± 1.00
T	0.25 ± 0.04*	6. 0± 0.87*	20.8 ± 2.33*	11.01 ± 2.33*	11.6 ± 0.32*
A/L	0.36 ± 0.06**	6.35± 0.45**	16.3 ± 1.71**	9.00 ± 0.41**	8.14 ± 0.71**
A/L/T	0.50 ± 0.03π	4.22± 0.27π	12.8 ± 0.79π	6.01 ± 2.70π	5.40 ± 0.26π

T: Tinidazole, A/L: Artemether/lumefantrine, AL/T: Artemether/lumefantrine/tinidazole MDA: Malondialdehyde, GSH: Glutathione, CAT: Catalase, SOD: Superoxide dismutase GPx: Glutathione peroxidase, Data as Mean ± SEM, n=5,* p<0.05 compared to control, **p<0.01 compared to NC, π p<0.001 compared to control, SEM: Standard error of mean.

3. RESULTS

3.1 Effects of artemether/lumefantrine/tinidazole on body, kidney weights and serum biochemical markers of parasitized mice

Treatment with T, A/L and A/L/T did not produce significant (p> 0.05) effects on body and kidney weights of parasitized mice when compared to normal control **(Table 1).** Normal (p> 0.05) serum electrolytes, creatnine, urea and uric acid, total protein and albumin levels, were observed in parasitized mice treated with T, A/L and A/L/T when compared to normal control **(Table 2)**.

3.2 Effects of artemether/lumefantrine/tinidazole on body, kidney weights and serum biochemical markers of healthy mice

Body weight was significantly decreased whereas kidney weight was significantly increased in healthy mice treated with T (p<0.05), A/L (p<0.05) and A/L/T (p<0.01) when compared to control **(Table 1)**. Serum creatnine, urea and uric acid levels were significantly increased whereas total protein and albumin levels were significantly decreased in healthy mice treated with T (p<0.05), A/L (p<0.01) and A/L/T (p<0.001) when compared to control **(Tables 3).** Serum electrolytes (Sodium, potassium, chloride, and bicarbonate) were normal (p>0.05) in healthy mice treated with T, A/L and A/L/T when compared to control **(Tables 3).**

3.3 Effects of artemether/lumefantrine/tinidazole on kidney oxidative stress markers and histology of healthy mice

Kidney antioxidants (SOD, GSH, CAT and GPx) were significantly decreased in healthy mice treated with T ($p<0.05$), A/L ($p<0.01$) and A/L/T ($p<0.001$) when compared to control **(Table 5).** On the other hand, MDA levels were significantly increased in healthy mice treated with T ($p<0.05$), A/L ($p<0.01$) and A/L/T ($p<0.001$) when compared to control **(Table 5).** Kidney of control mice shows normal histology **(Figure 1).** Kidney of healthy mice treated with A/L shows enlarged Bowman's space and tubular necrosis **(Figure 2).** Kidney of healthy mice treated with T shows tubular necrosis **(Figure 3).** Kidney of healthy mice treated with A/L/T shows enlarged Bowman's space and tubular necrosis **(Figure 4).**

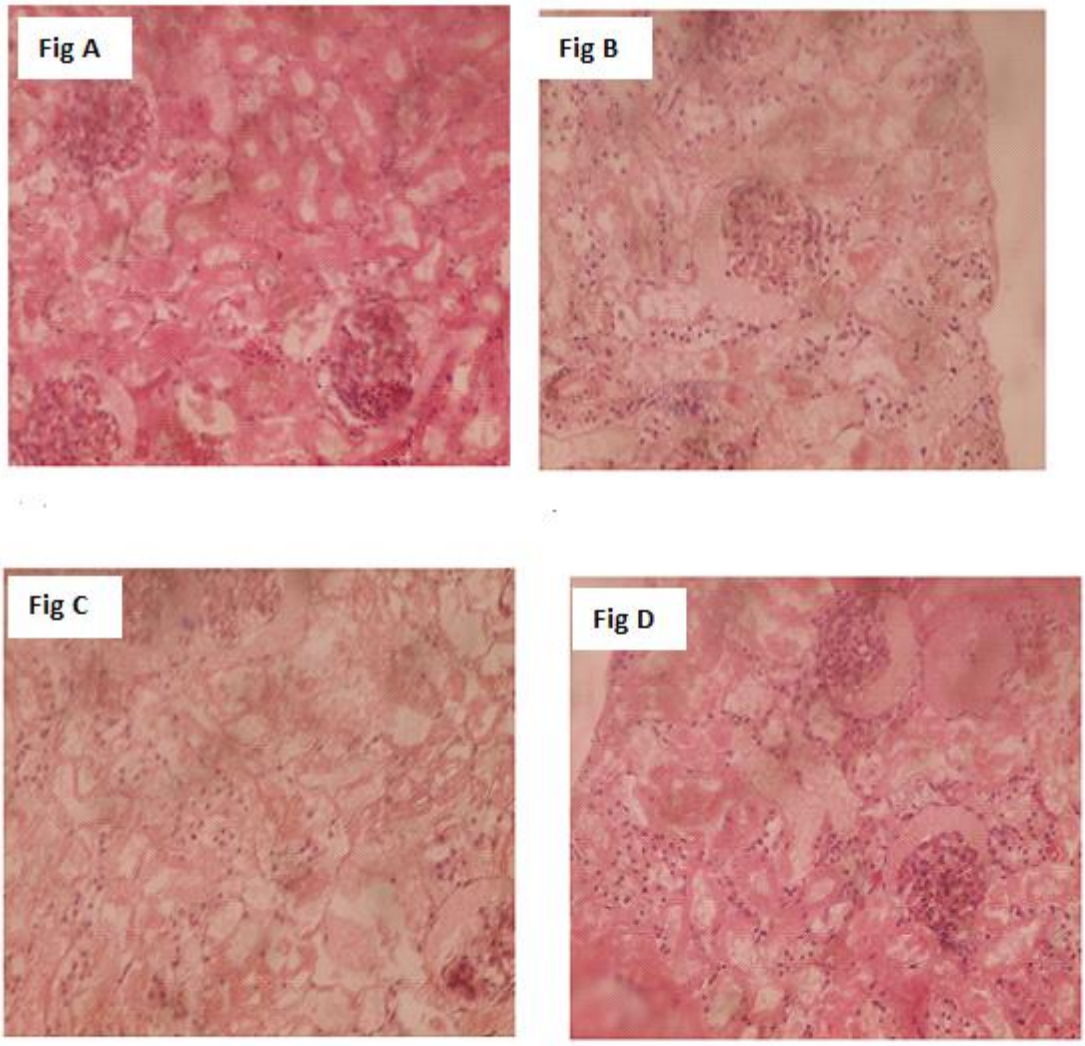

Fig 1: (A) Kidney of control mice shows normal histology. (B)Kidney of healthy mice treated with A/L shows enlarged Bowman's space and tubular necrosis. (C) Kidney of healthy mice treated with T shows tubular necrosis. (D) Kidney of healthy mice treated with A/L/T shows enlarged Bowman's space and tubular necrosis.

4. DISCUSSION

Previous study has shown the antiplasmodial activity of A/L/T in a mose model infected with *P. bergei* [14]. Thus study further assessed the safety of A/L/T on the kidneys of healthy and *P. bergei* infected mice. Perturbations in body and organ weights are used in experimental studies to ascertain the toxic effects of chemical substances [20]. In this study, body and organ weights were normal in healthy and parasitized mice treated with A/L/T. Biomarkers of renal function, are used to estimate the severity and nature of kidney injury, and to decipher appropriate therapy. Biomarkers can suggest the potential risk of kidney disease and the type of renal injury; by

predicting disease progression, as well as responses to therapies. Biomarkers including serum creatinine, urea and uric acid, total protein and albumin are use to assessed the extent of kidney perturbation. Creatinine gives a clear picture of glomeruli filtration rate whereas total protein can accurately detect early kidney perturbation as well as chronic kidney perturbation [21]. In this study, renal function markers were normal in parasitized mice treated with A/L/T whereas, acute toxicity study in healthy mice showed impaired renal function markers with A/L/T treatment. This was characterized by elevated serum creatinine, urea and uric acid levels with decreased total protein and albumin. Electrolytes are positively and negatively charged ions, which are found within cells and extracellular fluids, including intestinal fluid, blood, and plasma. Electrolytes play important functions in cellular function, intermediary metabolism, enzyme activities and electrical gradients [22]. Fluctuations in serum electrolytes have been used to assess kidney function [23]. The current study observed normal serum electrolytes in parasitized mice and healthy mice treated with A/L/T. Oxidative stress is a state in which oxidation exceeds the antioxidant systems which can cause hazardous damage to biomolecules. It can also alter physiologic adaptation phenomena and the regulation of intracellular signal transduction. Low levels of antioxidants have been experimentally used to assess oxidative stress [24]. Lipid peroxidation is a chain reaction by which unsaturated fatty acids (cell membrane components) are oxidized in various pathological conditions. Many markers of lipid peroxidation have been proposed, including lipid peroxides, malondialdehyde, and 4-hydroxynonenal [24]. The current study observed normal kidney antioxidants and malondialdehyde levels in parasitized mice treated with A/L/T. But in the acute toxicity study, healthy mice treated with A/L/T showed decreased antioxidants with increased malondialdehyde levels. This observation connotes oxidative stress. The kidneys of A/L/T treated healthy mice were distorted as marked by tubular necrosis and enlarged Bowman's space. The observation correlates with changes in serum renal function markers and kidney oxidative stress markers observed in A/L/T treated rats. A/L has been shown to cause nephrotoxicity characterized by alterations in serum creatinine urea and uric acid levels [25] which is consistent with the observation in this study. Also, changes in kidney oxidative stress markers and histology in A/L treated mice have been previously reported [25]. Studies have shown that T is relatively safe [11], but this study observed signs of nephrotoxicity in treated healthy mice characterized by altered kidney histology, and serum renal function indices. Also, in this study, treatment with T produced alterations in kidney oxidative stress markers of healthy mice.

Conclusion: The use of A/L/T as an antimalria may be safe on the kidney, but long term use may impair kidney function considering the doses and route used for this study.

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