EFFECTS OF ARTEMISININ-BASED COMBINATION THERAPY ON HISTOPATHOLOGY OF THE LIVER, KIDNEY AND SPLEEN OF MICE INFECTED WITH *PLASMODIUM BERGHEI*

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ABSTRACT

Malaria has remained one of the leading causes of morbidity and mortality in most developing countries. Artemisinin-based combination therapy (ACT) had been adopted for the management of the disease. This study evaluated the effects of therapeutic doses of artesunate + amodiaquine and dihydroartemisnin + piperaquine on the liver, kidney and spleen of mice infected with Plasmodium berghei. Sixty adult mice of eight weeks old with average weight of 22.5 ± 5.5 g were randomly divided into six groups of ten animals each. Plasmodium berghei was inoculated into the mice and observed for seven days, followed by three days oral administration of therapeutic doses of artesunate + amodiaquine (A&A) and dihydroartemisinin + piperaquine (D&P). Control groups were given water for the same period. Histopathology results revealed; periportal inflammatory cells, haemopoietic precursor cells, haemozoin pigmentation in the liver of the infected untreated and treated groups. The spleen showed haemozoin pigments, loss of the typical structure of the germinal centre, apoptotic lymphocytes with tinged macrophages, megakaryocytes and haemopoietic precursor cells in the infected untreated and treated groups. Inflammation of the renal pelvis was found in the kidney of the infected untreated group and the group treated with dihydroartemisinin + piperaquine. Cytoplasmic vacuolation was found in the liver after 28 days follow-up. Malaria infection and treatment with artesunate + amodiaquine (A&A) and dihydroartemisinin + piperaquine caused reversible damages to the liver, spleen and kidney.

Keywords: Malaria, Artemisinin, Liver, Spleen, Kidney, *Plasmodium berghei*

INTRODUCTION

Malaria is a serious and often fatal disease caused by malaria parasite of the genus *Plasmodium* (WHO, 2015). It has remained one of the leading causes of morbidity and mortality in most developing countries, especially in sub-Sahara region where the disease is endemic. It has remained a serious health challenge in Africa. Despite increasing efforts to reduce malaria infection and transmission, there has been little change in the areas at risk of the disease (WHO, 2015). In 2016, the World Health Organization recorded 216 million cases of malaria with an estimated 445,000 deaths (WHO, 2017). Since 2000, progress in reducing malaria burden in Africa has lagged behind that of other countries (WHO, 2015). Federal Ministry of Health reported that malaria was responsible for nearly 110 million clinical cases and estimated 300,000 deaths per year. It accounts for about 60 % of all outpatient attendance, 30 % of all hospital admissions, 25 % of death in children under one year and 11%

of maternal mortality (FMH, 2005a; FMH, 2015). It is one of the leading causes of avoidable death in children and pregnant women (Okorosobo *et al*., 2011; WHO, 2015). One of the major strategies to control malaria is prompt management with effective antimalarial drugs. However, due to malaria parasite resistance to chloroquine and other antimalarial drugs, newer antimalarial drugs have been discovered including artemisinin. Artemisinin is considered as a perfect replacement for chloroquine because it is a potent and rapidly acting blood schizonticide, eliciting shorter parasite clearance time and rapid symptomatic response than chloroquine and other antimalarial drugs (Qinghaosu Antimalaria Coordinating Research Group, 1979). Despite its efficacy, artemisinin has pharmacokinetic limitations. Naturally, artemisinin is not soluble in water or oil; it has poor bioavailability, and a short elimination half-life in vivo (~2.5 h) and high recrudescence rate of infection (Ashton *et al.,* 1998; Li *et al*., 2007). To overcome some of these problems, semisynthetic derivatives compounds of artemisinin have been developed, to improve the drug's pharmacological properties and antimalarial potency (Klayman*,* 1985).They include: artesunate, arteether, artemether, artemisone and dihydroartemisinin. These derivatives of artemisinin are more frequently used malaria chemotherapy, because of their effectiveness against *Plasmodium* parasite. The continued use of oral artemisinin-based monotherapies is considered to be a major contributing factor to the development of resistance to artemisinin derivatives. Therefore, the use of the drugs as monotherapy is explicitly discouraged by the World Health Organization (WHO, 2001). This has necessitated the use of combination therapy of artemisinin with other antimalarial agents known as the artemisinin – based combination therapies (WHO, 2006; Olliaro and Taylor, 2004). In 2001, the World Health Organization recommended the first-line use of artemisinin-based combination therapy (ACT). The five recommended ACTS are artesunate plus sulfadoxine plus pyrimethamine (sp), artesunate plus amodiaquine, artemether plus lumefantrine, artesunate plus mefloquine and dihydroartemisinin plus piperaquine (WHO, 2001). In 2005, artemisinin-based combination therapies (ACTs) were adopted as the first-line treatment for uncomplicated malaria in Nigeria (FMH, 2005b). This policy change was in line with global trends (WHO, 2001) and was hinged on demonstrated advantages of ACTs over chloroquine and sulfadoxine-pyrimethamine. ACTs are the mainstay of recommended antimalarial treatments today (Olliaro and Wells, 2009). Hence, the aim of this study was to determine the effects of artesunate + amodiaquine and dihydroartemisinin + piperaquine on histopathology of the liver, spleen and kidney of mice infected with *Plasmodium berghei.*

MATERIALS AND METHODS

Procurement of Experimental Animals: Animals used in this experiment were obtained from the Animal House of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

Experimental Design: The experiment was laid in a complete randomized design of six treatments replicated twice with each replicated having five mice of eight weeks old with average weight of 22.5 ± 5.5 g. These mice were randomly divided into six treatment groups. They were housed in separate cages, lined with sawdust beddings, fed on standard mice pellet diet, and given access to water *ad labitum*. The animals were allowed to acclimatize for one week before commencement of the study. All the animals used in this experiment were handled in accordance with the guidelines for ethical conduct and used of non-human animals in research as promulgated by APA (2002).

Experimental Treatment: *Plasmodium berghei* (NK 65) was inoculated to the mice via intraperitoneal route as described by Peter and Anatoli (1998) and Fidock *et al.* (2004). *Plasmodium berghei* infected red blood cells were obtained from the tail vein of the infected mice and diluted with 5 ml of phosphate buffered saline (PBS), so that 1 ml of parasitized blood contained 5 x 10^9 RBC m^{-1} infected

erythrocytes, each 0.2 ml of the blood that was subsequently injected contained 1 x 10^6 *Plasmodium berghei* parasitized red cells (Huang *et al.*, 2015). Degree of parasitaemia was determined using the method of Warhurst and Williams (1996). Parasite count in each of the groups of animals was determined at days 0, 3, 5 and 7. Drug administration commenced on day 8 post innoculation. Drugs were powdered separately in a mortar, mixed with known amount of distilled water and administered in mg/kg body weight as recommended by the WHO (2015) with oral gavage as follows- group A were infected but untreated (parasitized control), group B were infected and treated with 4 + 10 mg/kg of artesunate + amodiquine (A&A Group) for three days, group C were infected and treated with 4 + 18 mg/kg dihydroartemisinin + piperaquine (D&P Group) for three days, group D (A&A Recovery Group) and E (D&P Recovery Group) were infected and treated with 4 + 10 mg/kg of artesunate + amodiquine and 4 + 18 mg/kg dihydroartemisinin + piperaquine for three days respectively but were followed up to 28 days, group F were uninfected and untreated (normal control). After three days drug administration, animals were collected from groups A, B, C and F. After 28 days, animals were also collected from group D and E, they were anesthetized in chloroform vapour and dissected. The liver, spleen, and kidney were harvested and used for histopathological investigation.

Histopathological Analysis: This was carried out as described by Bancroft and Gamble (2002). The liver, spleen and kidney were fixed in 10 % formol saline and dehydrated in ascending grades of ethanol. Thereafter, the tissues were cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were later trimmed and sectioned at 5 microns. The sections were deparaffinized in xylene, mounted on clean slides, stained with Haematoxylin and Eosin (H and E) and examined under Olympus/3H light microscope. Photomicrographs were captured using a Moticam Images Plus 2.0 digital fitted to the light microscope.

RESULTS

The liver histopathology of the group infected and untreated showed remarkable periportal inflammatory cells infiltration, cytoplasmic vacuolation and pigmentations (haemozoin) (Figure 1).

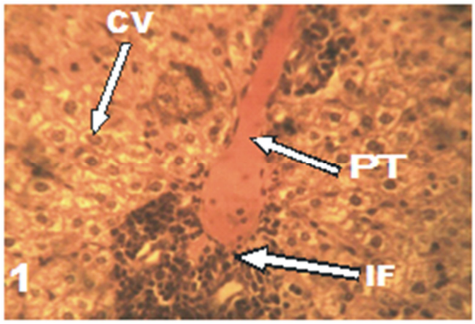

Figure 1: Section of the liver from the group infected and untreated showing remarkable periportal inflammatory cells infiltration (IF), pigmentations, cytoplasmic vacuolation (CV) and portal tract (PT). H and E, Mag. x 400

Group infected and treated with artesunate + amodiaquine showed minimal periportal inflammatory cells infiltration which was mixed with the presence of haemopoietic precursor cells (this is an indication of extramedullary haemopoiesis) and pigmentations (Figure 2).

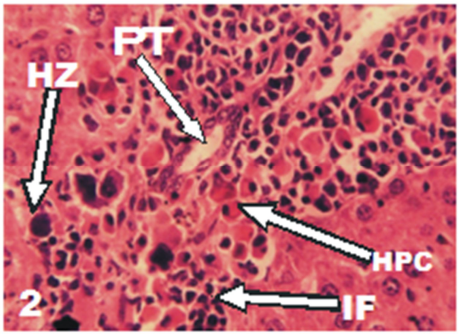

Figure 2: A section of the liver from mice infected and treated with artesunate + amodiaquine showing remarkable periportal inflammatory cells infiltration (IF), which was mixed with presence of haemopoietic precursor cells (HPC), haemozoin pigmentations (HZ), portal tract (PT). H and E, Mag. x400

Minimal periportal inflammatory cells infiltration was found in the liver of the group treated with dihydroartemisinin + piperaquine (Figure 3).

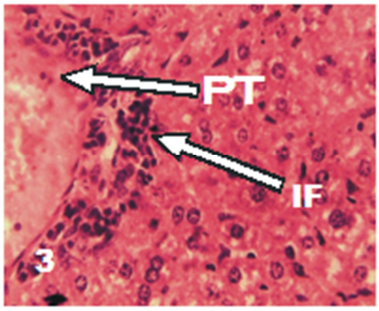

Figure 3: A section of liver from mice infected and treated with dihydroartemisinin + piperaquine showing minimal periportal inflammatory cells infiltration (IF), and normal portal tract (PT), H and E, Mag. x400

After 28 days follow-up, liver sections of the recovery groups showed normal portal tracts and cytoplasmic vacuolation (Figures 4 and 5).

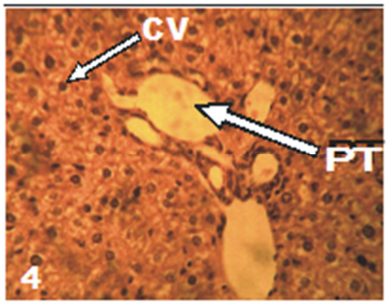

Figure 4: A section of liver from mice infected and treated with artesunate + amodiaquine and allowed to recover for 28 days, showing portal tract (PT) and cytoplasmic vacuolation (CV), H and E, Mag. x400

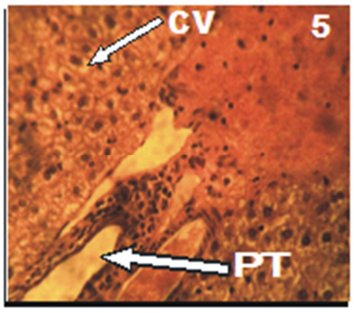

Figure 5: A section of liver from mice infected and treated with dihydroartemisinin + piperaquine and allowed to recover for 28 days, showing cytoplasmic vacuolation (CV) and normal portal tract (PT). H and E, Mag. x400

No histopathological changes were observed in the liver of the uninfected untreated group (Figure 6).

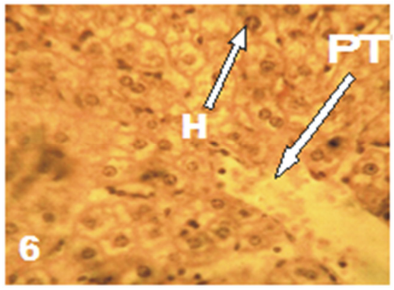

Figure 6: A section of liver from uninfected untreated group (normal liver) showing normal portal tracts (PT) and normal hepatocyte (H). H and E, Mag. x400

In the spleen, haemozoin pigments and macrophages were widely seen in the splenic sinusoids, in the infected untreated group (Figure 7), group treated with artesunate + amodiaquine (Figure 8) and the group treated with dihydroartemisinin + piperaquine (Figure 9).

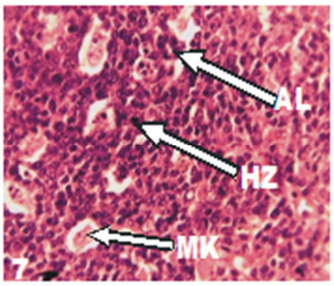

Figure 7: A section of the spleen from the infected untreated group, showing haemozoin pigments (HZ), apoptotic lymphocytes with tinged macrophages in the germinal centres (AL), wide spread megakaryocytes (MK), and other haemopoietic precursor cells within the red pulp H and E, Mag. x400

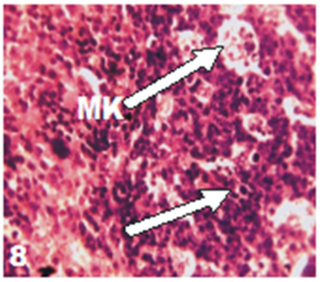

Figure 8: A section of the spleen from the group infected and treated with artesunate + amodiaquine for three days, showing haemozoin pigmentations and apoptotic lymphocytes with tinged macrophages in the germinal centers (AL), wide spread megakaryocytes (MK) and other haemopoietic precursor cells within the red pulp. H and E, Mag. x400

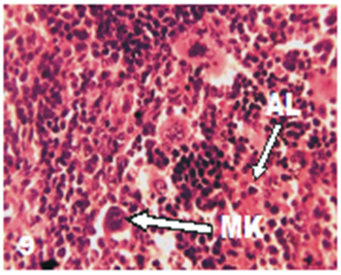

Figure 9: A section of the spleen from the group infected and treated with Dihydroartemisinin + piperaquine for three days, showing haemozoin pigments in the splenic sinusoids within the red pulp and apoptotic lymphocytes (AL) with tinged macrophages in the germinal centers. There were also wide spread megakaryocytes (MK) and other haemopoietic precursor cells within the red pulp. H and E, Mag. x400

There was a loss of the typical structure of the germinal center which was in these groups had apoptotic lymphocytes with tinged macrophage. Wide spread megakaryocytes and other haemopoietic precursor cells within the red pulp where also discovered in these groups.

However, their recovery groups showed traces of haemozoin pigments in the splenic sinusoids, apoptotic lymphocytes with tinged macrophages in the germinal center and presence of megakaryocyets (Figures 10 and 11). No histopathological changes were observed in the spleen of the uninfected untreated group (Figure 12).

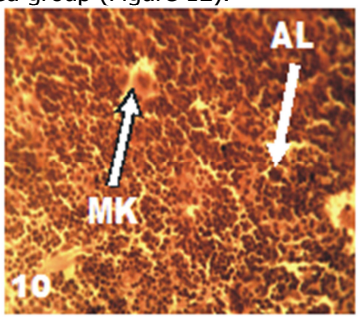

Figure 10: section of the spleen from the group infected and treated with artesunte + amodiaquine for three days, then followed up to 28 days, showing traces of haemozoin pigments in the splenic sinusoids, apoptotic lymphocytes with tinged macrophages in the germinal centres (AL), and presence of megakaryocytes (MK). H and E, Mag. x 400

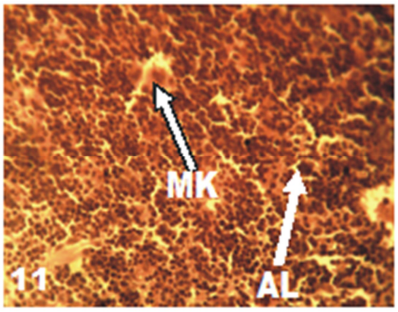

Figure 11: A section of the spleen from the group infected and treated with dihydroartemisinin + piperaquine and followed up to 28 days showing traces of haemozoin pigments in the splenic sinusoids, apoptotic lymphocytes (AL) with tinged macrophages in the germinal centers and presence of megakaryocytes (MK). H and E, Mag. x400

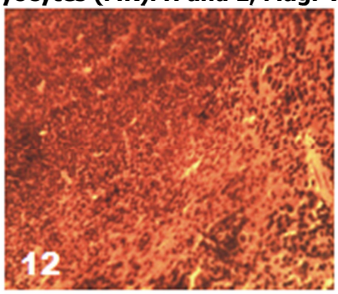

Figure 12: A section of the spleen from the infected untreated group (normal control) showing normal spleen architecture. H and E, Mag. x400

In the kidney tissues, inflammation of the renal pelvis was found in the infected untreated group (Figure 13) and the group treated with dihydroartemisinin + piperaquine (Figure 14).

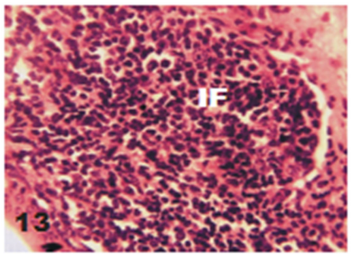

Figure 13: A section of the kidney from mice infected and untreated showing severe inflammation of the renal medulla/pelvis (IF). H and E, Mag. x400

Group treated with artesunate + amodiaquine (Figure 15) and all the recovery groups (Figures 16 and 17) showed no remarkable histological changes.

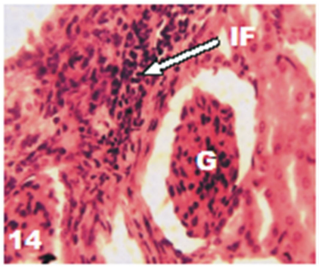

Figure 14: A section of the kidney from mice infected and treated with dihydroartemisinin + piperaquine for three days showing minimal inflammation (IF), normal glomerulus (G) and tubules. H and E, Mag. x400

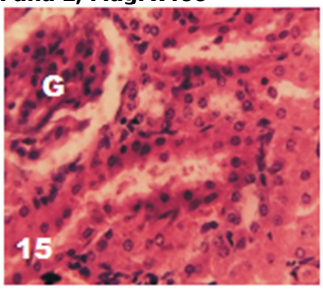

Figure 15: A section of the kidney from the group infected and treated with artesunate + amodiaquine for three days showing normal glomerulus (G) H and E, Mag. x400

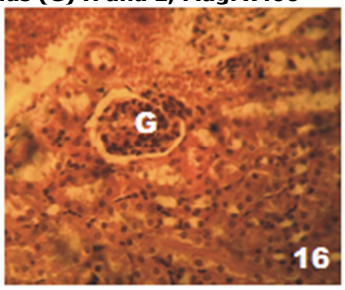

Figure 16: A section of the kidney from the group infected and treated with artesunate + amodiaquine for three days and allowed to recover for 28 days, showing normal glomerulus (G) and tubules. H and E, Mag. x400

No histopathological changes were also discovered in the kidney of the uninfected untreated group (Figure 18).

DISCUSSION

Histopathology of the liver revealed remarkable periportal inflammatory cells infiltration including the presence of haemopoietic precursor cells, deposition of malaria pigment (haemozoin) in both infected untreated and infected treated groups.

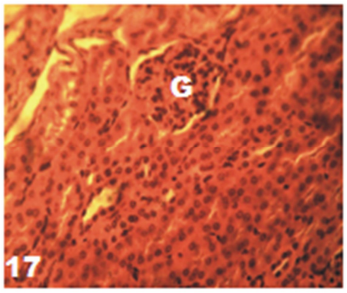

Figure 17: A section of the kidneyfrom the group infected and treated with and dihydroartemisinin + piperaquine and allowed to recover for 28 days showing normal glomerulus (G) and tubules. H and E, Mag. x400

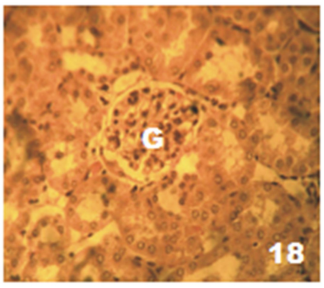

Figure 18: A section of kidney from the uninfected untreated group (normal control), showing normal glomerulus (G) and tubules. H and E, Mag. x400

Izunya *et al.* (2010) also reported mild inflammation of the portal tracts in the liver of rats treated with Artesunate. Their study suggested that artesunate at normal dose has a toxic effect on the liver cells and could be a potential hepatotoxic drug. These findings also agreed with the report of Onyije and Hart (2012) that discovered infiltration of inflammatory cells and loss of tissue architecture in the rats administered with 6 mg/kg of artesunate orally. The presence of haemopoietic precursor cells observed in the liver of the group treated with artesunate + amodiaquine is an indication of extramedullary haemopoiesis (blood cell formation in the liver). Extramedullary hematopoiesis is the proliferation of haematopoietic cells outside the bone marrow in response to the production of too few blood cells to satisfy the body's demand (Choi *et al.*, 2004). This insufficient production is caused by either bone marrow replacement disease or hemolytic anemia (Choi *et al.,* 2004). Where extramedullary haematopoiesis involves an organ, there is usually radiographic evidence

of its enlargement (Choi *et al*., 2004). This may contribute to liver and spleen enlargement observed in this experiment. However, all the hepatotoxicities observed in the infected treated groups were not observed in their recovery groups. Therefore, malaria infection and its treatment with artesunate + amodiaquine and dihydroartemisinin + piperaquine induced reversible effects in the liver of mice. The liver is susceptible to these toxicities because all the foreign substances and drugs are metabolized and inactivated in the liver. The organ is also involved during the hepatic stage of malaria where malaria sporozoites developed into merozoites (Adachi *et al*., 2001).

In the spleen, haemozion was also observed in the infected groups. This organ is the site for the breakdown and removal of abnormal or worn-out red blood cells. Therefore, the spleen also contributed to the accumulation of hemozion pigments molecules which was also noticed in the liver of the infected groups. The widespread of malarial pigments was found to be consistent with the elevated parasitaemia level in the infected mice; higher pigmentation could further impair the macrophage function (Helegbe *et al*., 2011) and trigger the host immune system to release more cytokines (Turrini *et al*., 1993). The release of pro-inflammatory cytokines may have caused splenic tissue abnormalities as observed in the treated mice. Loss of the typical structure of the germinal center was also observed in the spleen of all the infected rats. This finding was in line with the report of Basir *et al.* (2012). Apoptotic lymphocytes with tinged macrophages in the germinal centers were observed in the spleen. Apoptosis is characterized by shrinkage of individual lymphocytes, condensation of nuclear chromatin, and fragmentation of apoptotic cells into membrane-bound bodies (apoptotic bodies, which are subsequently phagocytized by macrophages (tangible body macrophages) (Kapoor *et al*., 2011). There were also widespread megakaryocytes and other hamopoeitic precursor cells within the red pulp in all the infected groups. Megakaryoblast is a precursor cell to a megakaryocyte during haematopoiesis. The presence of haemopoietic precursor cells observed in the spleen is an indication of extramedullary haemopoiesis as found in the liver. Extramedullary hematopoiesis is the formation and development of blood cells outside the medullary spaces of the bone marrow (Johns and Christopher, 2012). It occurs most often in the spleen in association with degenerative and inflammatory conditions, including lymphoid hyperplasia, hematomas, and thrombosis (Ballegeer *et al*., 2007). Yin *et al*. (2014) reported that intramuscular administration of 6 mg kg^{-} artemether over a 3 months period induced concurrent extramedullary hematopoiesis in the spleen and inhibition of erythropoiesis in the bone marrow of dogs. However, traces of haemozoin pigments in the splenic sinusoids were observed after the recovery period.

In the kidney, inflammation of the renal pelvis was observed in the infected untreated group and the group treated with dihydroartemisinin + piperaquine. Inflammation is a vital part of the body's immune response. Inflammation of the renal pelvis is most commonly associated with an infection. It was hypothesized that the release of malaria antigens activates monocyte cells, to release pro-inflammatory cytokines and activate cell-mediated response, causing renal problems (Barsoum, 1998). Artemisinins are selectively distributed into *P. falciparum* infected erythrocytes, where they cause malaria parasite's death through the generation of free radicals (Vyas *et al*., 2002; Little *et al*., 2009). However, these drugs are also distributed in other organs including the liver, CNS, lungs, kidney and spleen (Zhao and Song, 1989; Vyas *et al*., 2002) making such organs possible targets of toxicity.

Conclusion: This study demonstrated that malaria infection and treatment with artesunate + amodiaquine and dihydroartemisinin + piperaquine were toxic to the liver, kidney, and spleen. The liver and spleen were more affected than the kidney. There were signs of recovery after 28 days follow up. Therefore these drugs should be used with caution especially in patients with previous history of liver, spleen and kidney impairments.

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