Evaluation of the Effects of Artesunate on the Liver of Plasmodium Berghei Infected Mice

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ABSTRACT

Background: Antimalaria drugs play important role in treating malaria but some medicinal agents when taken in overdoses or even within therapeutic ranges maybe toxic. Artesunate is one of the successfully used over-the-counter antimalaria
drug in treating and preventing malaria but many studies have shown validation of toxicity on several organs. The aim of this study was to investigate the effect of Artesunate on the liver of Plasmodium berhei infected mice. Methods: 25 mice were divided into 5 groups of 5 animals each, Group 1 received 0.5 ml/kg distilled water, Group 2 received 4 mg/kg/day Artesunate only, Group 3 was inoculated with Plasmodium berghei only, Group 4 and 5 were inoculated with P. berghei and treated with 4 and 8 mg/kg/day Artesunate respectively 72 hours after inoculation. The treatment lasted for a period of 5 days after which the mice were humanly sacrificed. Blood samples were collected by cardiac puncture and stored in 5 ml plain and EDTA bottles for biochemical analysis. The liver tissues were removed and placed in neutral buffered saline.

RESULTS: There was no significant difference in the liver function biomarkers (ALT, AST and ALP) examined when compare among the groups. There was also significant increase in SOD activities in Group 5 when compared with Group 1, 2, 3 and 4, also there was a significant decrease in CAT and GSH activities in Group 1 when compared with Group 3, 4 and 5, the MDA activities in Group 5 showed a significant increase when compared with the other Group. There was increase in the liver weight and liver somatic index of Group 3 when compared to Group 1. Histological changes were observed in Group 2, 3, 4 and 5 when compared with Group 1. This research shows that Artesunate and malaria has adverse effect on the liver. There is need for caution during the administration of Artesunate in the treatment of malaria.

CONCLUSION: The effects of Artesunate on the liver of Plasmodium berghei infected mice were evaluated in this study. The results obtained show that Artesunate induces oxidative stress in mice infected with Plasmodium berghei and causes cytoplasmic vacuolation, traces of periportal mononuclear cells infiltration, and severe and diffused hepatic necrosis with karyolysis in the liver of mice infected with Plasmodium berghei.

INTRODUCTION

Malaria is a preventable and treatable disease. The primary objective of treatments is to ensure complete cure that is the rapid and full elimination of the Plasmodium parasite from the patient’s blood, in order to prevent progression of uncomplicated malaria to severe disease or death and to prevent chronic infection that leads to malaria-related anaemia (WHO 2018).

Artemisinin is currently the most widely used antimalarial drug against drug-resistant malaria. It is a Chinese herb that has been used over thousands of years for the treatment of fever. It is extracted from the leaves of Artemisia annua (sweet wormwood). Artemisinin and its derivatives (artesunate, artemether, arteether and dihydroartemisinin) are highly potent, rapidly eliminated antimalarial drugs with a broad stage specificity of action. They clear parasitemia more rapidly than all other currently available antimalarial agents. Artesunate is a
hemisuccinate derivative of the active metabolite dihydroartemisin. Currently it is the most frequently used of all the artemisinin-type drugs. Its only effect is mediated through a reduction in the gametocyte transmission. It is used in combination therapy and is effective in cases of uncomplicated P. falciparum (Izunya et al., 2010; Ashley et al., 2014).

The liver is a vital organ only found in vertebrates (Abdel-Misih and Bloomston, 2010). The liver is a reddish-brown wedge-shaped organ with four lobes of unequal size and shape. A human liver normally weighs 1.44 – 1.66 kg (3.2 – 3.7 Ib.), and has a width of about 15 cm, it is both the heaviest internal organ and the largest gland in the human body. Located in the right upper quadrant of the abdominal cavity, it rests just below the diaphragm, to the right of the stomach and overlies the gallbladder. The numerous functions of the liver are carried out by the liver cells. The liver is thought to be responsible for up to 500 separate functions usually in combination with other systems and organs. It plays a role in metabolism, regulation of glycogen storage, decomposition of red blood cells and hormone production. It also helps in detoxification of various metabolites, protein synthesis, and the production of biochemical necessary for digestion (Tortora and Derrickson, 2008).

Artesunate is one of the successfully used over-the-counter antimalarial therapies in treating and preventing malaria, Many studies on toxicity of Artesunate have shown validation of toxicity on the stomach, brain stem, and the liver. Administration of Artesunate for the treatment of malaria maybe an additional burden on the liver which is already undergoing insult from malaria parasite invasion of the hepatocytes, this therapy even in normal doses may result in severe hepatic injury to an already damaged organ. Some studies have suggested evidence of toxicity on the brainstem, superior colliculus, stomach, testis and liver in artesunate treated rats (Maude et al., 2009). Moreover, artesunate has been reported to destroy cancer cells and also reduces proliferation, interferes in DNA replication and cell cycle and enhance apoptosis through the intrinsic death pathway by ROS generation. It has been reported that artesunate is toxic at nanomolar concentration to malaria parasites, while micromolar concentration produced toxicity in mammalian cells (Turschner and Efferth, 2009). Evidences showed the neurotoxicity of artesunate at high doses (50 – 100 mg/kg per day oral and IM) in laboratory animals (Brewer et al., 1994) including the cytotoxicity of artesunate on tumor cell lines have been reported (Maude et al., 2009). Therefore, this research was carried out further help in understanding the adverse effect of Artesunate and malaria on the liver and also provide the need for caution during the administration of Artesunate in the treatment of malaria.

**MATERIALS AND METHODS:**

Reagents: Randox test kit, Microscope, glass slides and cover slips, Formalin
and bottles for fixing tissues, Dissecting set, Beakers and syringes, Anaesthesia (Ketamin), Normal saline, Haematoxylin and eosin. Experimental Design: The animals were grouped into five groups of five (5) male mice per group. One control group and four treated groups; group III to V were inoculated with malaria on the first (1st) day of the experiment. Group I served as the control group and received 1 ml/kg normal saline intraperitoneally during the first day of the experiment and were given distilled water orally subsequently for a period of 5 days, Group II served as the Artesunate group and received 1 ml/kg normal saline intraperitoneally during the first day of the experiment and was latter administered with 4 mg/kg body weight of Artesunate starting from the 4th (fourth) day of the experiment, Group III served as the Untreated malaria infection groups while Group IV, and V were treated malaria infection group in different doses; the animals were infected with malaria and treated with Artesunate at 4 mg/kg body weight, and 8 mg/kg body weight respectively. Malaria infection was established on the 3rd day before commencement of treatment. The mice were treated with Artesunate for five (5) days before sacrifice. Artesunate was dissolved in distilled water and administered orally with the aid of a stainless metallic feeding cannula. The animals were then humanely sacrificed under anesthesia using Ketamine, through a midline incision on the thoracic wall, the heart was accessed and blood samples were collected by cardiac puncture. The blood samples were stored in 5 ml plain bottle for biochemical evaluation. The liver was quickly excised, rinsed in normal saline, gently blotted between folds of a filter paper and then weighed using an electronic compact digital scale. The liver tissue was cut into two pieces; the first piece was immediately fixed in Neutral Buffered Formalin for subsequent histological and histochemical analysis. The second piece was weighed and homogenized in Phosphate Buffer (pH 7.4) (at a ratio of 1 g to 5 ml respectively) for estimation of Oxidative stress parameters. The liver Enzymes and Oxidative Biomarkers were determined by ELISA technique (Kavishe et al., 2017). The tissues were Processed with Tissue Processing Machine and stained using haematoxylin and eosin (H and technique for general liver histology, Statistical Analysis: Data obtained were expressed as mean ± SEM (Standard Error of Mean). One-way analysis of variance (ANOVA) was used to compare the mean differences followed by LSD post-hoc test, P-value less than 0.05 was considered to be statistically significant. All the results were analyzed using the Statistical Package (SPSS version 20)

RESULTS AND DISCUSSION:

This study revealed that there was no significant difference in the liver weight and liver somatic index when compared between the Groups, although there was increase in the liver weight and liver somatic index of Group 3 when compared to Group 1 (Table 1.0). Group 4 also showed a slight increase in the liver weight and liver somatic index when compared to Group 1, Group 5 showed increase in the liver weight and liver somatic index when compared to Group 1 but it was not
significant statistically (Table 4.2). Rungruang et al (2013) reported that the liver of Plasmodium infected mice showed no significant difference in weight when compared with the control, hepatomegaly was observed on day 5 and 6 post-infection of Swiss Albino mice infected with Plasmodium yoelli nigeriensis (Ahmad and Srivastava, 2007). The reason for increase in liver weight and liver somatic index could be that the liver of infected mice is under very severe condition; part of systemic sequestration and anemia reduce the chance of circulating cells passing into the organs (Thuma et al., 1998).

There were no statistical differences (p<0.05) in all the liver injury biomarkers: Alanine transaminase (ALT), Aspartate aminotransferase (AST), Alanine phosphate (ALP) investigated between the experimental groups and the control groups. However, there was a slight increase in the ALT of Group 3 (128.00±20.06) when compared to other groups, there was also a slight decrease in ALT of Group 2 (106.75±15.34) when compared to the other groups (Table 1.0).

There was also no statistically significant difference (p<0.05) in the AST of the all the experimental groups when compared to the control groups (Table 1.0). There was slight increase in AST of Group 4 when compared to other groups. The AST activities of Group 2 were lower when also compared with the other groups.

This investigation also revealed no statistically significant difference (p<0.05) in the ALP of all the groups when compared. Group 5 (155.40±25.52) showed the highest level of ALP when compared with the other Groups. Group 4 (148.40±49.36) showed a slight increase in ALP level when compared with Group 1 (137.25±32.42) and Group 2 (142.00±52.38).

Liver function tests are groups of blood tests that give information about the state of the liver (Lee, 2009). Injury to the liver may affect the integrity of hepatocytes leading to the release of liver enzymes such as alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphate (ALP) since these enzymes are confined to hepatocytes and released into the blood following liver injury. Hence, these enzymes are commonly used as markers of hepatic injuries (Ozer et al., 2008). The liver aspartate transaminase (AST) and alanine transaminase (ALT) are useful biomarkers of liver injury with some degree of intact liver function (Mengel and Schwiebert, 2005). These tests can be used to detect the presence of liver disease, distinguish among different types of liver disorders, gauge the extent of known liver damage and follow the response to treatment. In the present study, there was no significant difference statistically in serum level of AST, ALT and ALP as compared between the groups.

The level of liver dysfunction may be determined by the level of parasitemia in the body (Onyesom and Onyemakonor, 2011). Hamman et al (2011) also reported no statistical significant differences in ALP, AST and ALT levels in Wistar rats that were administered Artesunate at various doses.
The result revealed statistically significant (p<0.05) increase in Superoxide Dismutase (SOD) activities in all the groups; Group 4 (21.24±1.35); Group 5 (24.80±1.64) when compared to Group 1 (17.22±0.84) and Group 2 (16.58±0.45). Group 5 (24.80±1.64) also showed a significant statistical (p<0.05) increased in the SOD activities when compared to Group 1 (17.22±0.84), Group 2 (16.58±0.45), Group 3 (19.52±0.96) and Group 4 (21.24±1.35) There was significant statistical increase in Catalase (CAT) activities in Group 5 (10.14±0.50); Group 4 (10.24±0.39); and Group 3 (9.92±0.36) when compared with Group 1 (7.96±0.43) and Group 2 (8.00±0.33). There was also slight decrease in CAT activities in Group 1 (7.96±0.36) when compared to Group 2 (8.00±0.33) however it was not statistically significant (Table 2.0).

There was significant statistical increased in Glutathione (GSH) activities in Group 5 (38.16±2.97); Group 4 (43.88±1.81); and Group 3 (39.40±2.54) when compared with Group 1 (25.06±2.18) and Group 2 (31.04±1.43). There was also slight decrease in GSH activities in Group 1 when compared to Group 2 but was statistically insignificant (Table 2.0).

There was significant statistical increase in the Malondialdehyde (MDA) activities in Group 5 (37.68±3.15) when compared with other groups. There was also significant statistical increase in the MDA activities in Group 2 (34.44±2.75) when compared to Group 3 (25.58±1.94). This result also showed slight decrease in MDA activities in Group 3 (25.58±1.94) when compared to Group 1 (27.68±1.29) and Group 4 (29.06±2.08), however it was statistically insignificant (p<0.05) (Table 2.0).

The role of oxidative stress during malaria infection is still unclear. Some researchers argued a protective role, whereas other claims a relation to the pathophysiology of the disease (Sohail et al., 2007). However, other studies suggested that the generation of reactive oxygen and nitrogen species (ROS and RNS) associated with oxidative stress, plays a critical role in the development of systemic complications caused by malaria. Malaria infection induces the generation of hydroxyl radicals (OH-) in the liver, which most probably is the main reason for the induction of oxidative stress and apoptosis (Guha et al., 2006). The iron-rich environment within the parasite enables rapid production of specific redox active drug molecules and perhaps ROS that can lead to a rapid reaction and destruction of several specific systems within the parasite such as components of cellular membranes, the redox systems of the parasites, and the mitochondrial electron transport chain, and this may explain the rapid elimination of parasites by Artesunate (Kavishe et al., 2017).

In the present study there was significant increase in the GSH activities in Group 3, Group 4 and Group 5 when compared to Group 1 and Group 2 (Table 4.4). In Plasmodium parasites, GSH is also involved in the degradation of the toxic ferriprotoporphyrin IX (FP IX), which escapes from hemozoin formation.
Additionally, GSH functions as an electron donor for the enzyme ribonucleotide reductase (RNR), and also crucial for DNA synthesis and cellular proliferation (Buchholz et al., 2010). Artesunate may induce DNA double-strand breaks in Plasmodium infection in a physiologically relevant dose and time dependent manner which is accompanied by an increase in the ROS level in the parasites (Gopalakrishnan and Kumar, 2015). Mannitol, a ROS scavenger, reversed the cytotoxic effect of Artesunate and reduced DNA damage and modulation of GSH activities impacted ROS and DNA damage induced by Artesunate (Gopalakrishnan and Kumar, 2015). The present study also showed no statistically significant difference between the group that was inoculated with malaria but not treated and the groups that were treated with Artesunate after malaria inoculation, it was also discovered that at increased dose of Artesunate there was reduce GSH activities when compared with the malaria treated group. This could occur because Plasmodium possesses two redox systems which are the thioredoxin and the glutathione system, glutathione is the most abundant low molecular weight redox active thiol in the parasites existing primarily in its reduced form representing an excellent thiol redox buffer. This allows for an efficient maintenance of the intracellular reducing environment of the parasite cytoplasm and its organelles. During development, malaria parasites are exposed to environmental and metabolic stresses.

H&E stain result of the liver sections from Group 1 showed normal cytoarchitecture of the liver parenchyma; the characteristic appearance of the hepatocytes radiating from the central vein. Fine vascular spaces separate the thin plate of hepatocytes, the sinusoids and Kupffer cells found within the sinusoid lining (Plate I). Liver section from Group 2 showed some abnormal morphological characteristics showing Cytoplasmic Vacuolation (CyV) and Sinusoidal Congestion (SC) (Plate II). Liver section from Group 3 showed Cytoplasmic Vacuolation (CyV) with Kupffer cells Hyperplasia (KH), Central vein congestion (CCV) and Hemozoin (H) (Plate III). Liver section from Group 4 showed Cytoplasmic Vacuolation (CyV), Sinusoidal congestion (SD) and traces of Periportal Mononuclear cells infiltration (PM) (Plate IV). Liver section from Group 5 showed distortion in the cytoarchitecture with severe and difused Hepatic Necrosis with karyolysis (HN), Cytoplasmic vacuolation (CyV), traces of Periportal Mononuclear cells infiltration (PM) and Sinusoidal Congestion (SC) (Plate V).

**Table 1 Liver functions biomarkers**

<table>
<thead>
<tr>
<th>Groups Treatments</th>
<th>ALT (IU/L) Mean±SEM</th>
<th>AST (IU/L) Mean±SEM</th>
<th>ALP (IU/L) Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Distilled water</td>
<td>110.25±10.05</td>
<td>77.00±17.31</td>
<td>137.25±32.42</td>
</tr>
<tr>
<td>2 Artesunate 4 mg/kg/day only</td>
<td>106.75±15.34</td>
<td>65.00±11.79</td>
<td>142.00±52.38</td>
</tr>
<tr>
<td>3 Malaria Induced (Untreated)</td>
<td>128.00±20.06</td>
<td>83.75±24.33</td>
<td>153.00±33.52</td>
</tr>
<tr>
<td>4 Malaria Induced and treated</td>
<td>125.40±23.98</td>
<td>83.40±13.78</td>
<td>148.40±49.36</td>
</tr>
</tbody>
</table>
Table 2. Oxidative stress markers

<table>
<thead>
<tr>
<th>Groups Treatments</th>
<th>SOD(U/ml) Mean±SEM</th>
<th>CAT(U/mg) Mean±SEM</th>
<th>GSH(ug/ml) Mean±SEM</th>
<th>MDA(nMols/mg) Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Distilled water</td>
<td>17.22±0.84ae</td>
<td>7.96±0.43ace</td>
<td>25.06±2.18ace</td>
<td>27.68±1.29a</td>
</tr>
<tr>
<td>2 Artesunate 4mg/kg/day only</td>
<td>16.58±0.45bf</td>
<td>8.00±0.33bdf</td>
<td>31.04±1.43bdf</td>
<td>34.44±2.75bd</td>
</tr>
<tr>
<td>3 Malaria Induced (Untreated)</td>
<td>19.52±0.96c</td>
<td>9.92±0.36ab</td>
<td>39.40±2.54ab</td>
<td>25.58±1.94d</td>
</tr>
<tr>
<td>4 Malaria Induced and treated 4mg/kg/day Artesunate</td>
<td>21.24±1.35def</td>
<td>10.24±0.39cd</td>
<td>43.88±1.81cd</td>
<td>29.06±2.08c</td>
</tr>
<tr>
<td>5 Malaria Induced and treated 8mg/kg/day Artesunate</td>
<td>24.80±1.64abcd</td>
<td>10.14±0.50ef</td>
<td>38.16±2.97ef</td>
<td>37.68±3.15abc</td>
</tr>
</tbody>
</table>

f 8.693 8.183 10.930 4.614
p <0.001 <0.001 <0.001 0.008

One-way ANOVA test followed by Tukey post hoc test; Results expressed as mean±SEM, cells carrying same superscripts on each column are significantly different (p<0.05). SOD: Superoxide Dismutase; CAT: Catalase; GSH: Glutathione; MDA: Malondialdehyde.
Plate I: Transverse section of the Liver from Group 1
showing normal cytoarchitecture; Hepatocytes (H) Central Vein (CV), Sinusoid (S) and Kupffer Cells (K).
(H&E x250).

Plate II: Transverse section of the Liver from Group 2
showing the Central Vein (CV), Cytoplasmic Vacuolation (CyV) and Sinusoidal Congestion (SC) (H&E x250).

Plate III: Transverse section of the Liver from
Group 3 showing Cytoplasmic Vacuolation (CyV) with Kupffer cells Hyperplasia (KH), Central vein Congestion (CCV), Hemozoin (H) (H&E x250).
PLATE V: Transverse section from the Liver from Group 5 showing distortion in the cytoarchitecture with severe and diffused Hepatic Necrosis with karyolysis (HN), and Sinusoidal Congestion (SC), Cytoplasmic vacuolation (CyV) (H&E x250)

CONCLUSION: This research shows that Artesunate and malaria has adverse effect on the liver. There is need for caution during the administration of Artesunate in the treatment of malaria.

REFERENCES

Artesunate and its effect on the hematological indices, hepatotoxicity and histology of the liver of adult Wistar rats. Asian Journal of Medical Sciences 3(4):176 – 179
