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HISTOLOGICAL AND BIOCHEMICAL EFFECTS OF ARTEETHER™ ON THE LIVER OF WISTAR RATS

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Abstract

Arteether™ is among the recent drugs that are used to combat chloroquine-resistant malarial parasites. This study examined the effects of arteether™ on enzyme biomarkers of the liver, serum protein concentrations, and liver morphology. Twenty (20) adult albino Wistar rats weighing 200 – 250 g were randomly divided into four groups (A, B, C and D) of five animals each, and used in this study. Group A rats were given intramuscular (i. m.) arteether™ (3 mg/kg b. w.) daily for 3 days. Group B rats received i. m. arteether™ (6 mg/kg b. w.) daily for 3 days. Group C rats were given i. m. arteether™ (3 mg/kg b. w.) daily for 3 days. The same dose was repeated at two-weekly intervals for 4 further weeks, while group D rats which received normal saline (0.9 % w/ v, 3 ml/kg b.w.), served as controls. At the end of the experiment, the body weights of the animals were determined and recorded. Serum levels of alanine transaminase (ALT), aspartate transaminase (ASP), alkaline phosphatase (ALP), total protein (TP) and albumin were assayed, and histological studies were performed. Results obtained show no significant difference ($P<0.05$) in liver enzymes (ALT, ASP, ALP). TP and albumin were significantly reduced in group C rats. Histological studies revealed no cyto-architectural changes. It is concluded that at therapeutic doses, arteether™ is well tolerated in Wistar rats. .

Key Words: Arteether™; Malaria; Liver enzymes; Serum protein concentrations; Morphology; Wistar rats

Introduction

Malaria is endemic in most tropical and subtropical regions of the world. It is estimated that 300–500 million people are at risk of contracting malaria, with about 900,000 new cases diagnosed each year (Murray and Lopez, 1994; Olliaro et al., 1996). There are 1–2 million deaths reported annually due to severe malarial attack, with the majority of these deaths being children in Africa (Zucker and Campell, 1993).

In the face of resistance to chloroquine by *Plasmodium falciparum*, the malarial parasite responsible for the high mortality and morbidity from malarial disease, artemisinin and its derivatives (artesunate™, artemether™, arteether™, and dihydroartemisinin) have given renewed hope for combating resistant strains of *Plasmodium falciparum* malaria (Hien, 1994; Harinasuta and Karbwang, 1994). Artemisinin and its various derivatives are derived from the herb *Artemisia annua*, a herb known in China as 'qinghao' (Chinese word for "from green herb").

Artemisia annua is a member of the 'Asteraceae' family. It is native to Asia and has been used as antiparasitic therapy for malaria in Traditional Chinese Medicine (TCM) for more than 1,000 years (Chris, 2004). The 'explosive' nature of the mechanism of action of artemisinin and its derivatives is of interest and has given rise to the hope that it could prevent any rapid occurrence of plasmodial resistance. However, formation of free radicals has a potential for serious adverse reactions (Chris, 2004). Surprisingly, artemisinin and its derivatives only have few adverse effects, compared to chloroquine and many other older antimalarial drugs. Regimens of artemisinin derivatives given to patients with acute *falciparum* malaria as single agents have been associated with the following mild side-effects: acute nausea (16%), vomiting (11%), anorexia (34%), and dizziness (15%). Nevertheless, concerns have been expressed centering on possible neurotoxicity of artemisinin and its derivates (Brewer et al., 1994). In all experimental mammals tested (rats, dogs, primates), intramuscular injections of the oil-soluble antimalarial artemisinin derivatives, artemether™ and arteether™, have produced an unusual pattern of selective damage to brain-stem centres, predominantly involved in auditory processing and vestibular reflexes. Neurological findings included gait disturbance, loss of spinal and pain response reflexes, and prominent loss of brain stem and eye reflexes. It has been reported that artemether™ produces dose-dependent neuropathological damage to the brain-stem in the mouse (Nontprasert et al., 2002). The neurons in the lower brain-stem trapezoid nucleus, the *gigantocellular reticular* nucleus, and the inferior cerebella peduncle were the most sensitive to the toxic effects of artemether™ (Nontprasert et al., 2002). Most importantly, it has been shown that *in vitro*

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artemisinin induces oxidative stress in cultured neurons, as indicated by an increase in reactive oxygen species and extensive lipid peroxidation (Schmuck et al., 2002). Artemether™ has also been shown to induce transient and moderate elevations in liver transaminases and bradycardia (Nwanjo, et al., 2007).

Although artemisinin derivatives have been reported to be relatively safe, there is a paucity of literature on the effects of arteether™, a semi-synthetic derivative of artemisinin, on the structure and activities of the liver, which metabolises artemisinin derivatives including arteether™. Thus, the present study examined the effects of arteether™ on liver morphology and the enzyme biomarkers of the liver in Wistar rats.

Materials and Methods

All experiments were conducted in strict compliance with the humane animal care standards outlined in the "Guide to the Care and Use of Laboratory Animals in Research and Teaching" prepared by the National Institutes of Health (NIH), publication 86-23 (revised in 1985).

Animal Management

Twenty healthy albino Wistar rats (*Rattus norvegicus*) of both sexes (weighing between 100 g and 150 g) were obtained from the Department of Anatomy, Ladoke Akintola University of Technology, Ogbomoso, Nigeria, and used for this study. There was a pre-experimental period of four weeks during which the animals acclimatised and were maintained on standard rat chow and given tap water *ad libitum* before the commencement of our experiments. The body weights of the animals increased to between 200 g and 250 g before the commencement of our experiments. The animals were randomly assigned into four groups (A, B, C, and D) of five rats each. Each group was kept in a separate cage under natural light and dark cycles at room temperature. Group A rats were given intramuscular arteether™ (3 mg/kg b. w.) daily for 3 days. Group B rats received i.m. arteether™ (6 mg/kg b. w.) daily for 3 days. Group C rats were given (3 mg/kg b. w.) daily for 3 days (and the same dose was repeated at two-weekly intervals - making 3 consecutive treatments), while group D rats (which served as 'control animals') received i. m. equivalent volume of normal saline daily for three days. The animals received humane care and treatment according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (National Academy Press, 1996).

Specimen Collection

At the end of the experimental period, the animals were observed for general physical activity, mobility and agility. The body weights of the animals were taken, using Bharti spring balance. They were sacrificed by cervical dislocation. The thorax of each animal was opened through a midline incision, and the right ventricle was punctured for venous blood sample collection. Serum for biochemical analyses was obtained after centrifuging the blood samples for 5 minutes at 5000 rpm in a Denley BS400 centrifuge (England). The organ of study (liver) was excised and fixed in 10% formol saline for histological studies.

Biochemical analysis
Determination of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST)

ALT and AST in serum were determined using Randox kits (Randox Laboratory, Northern Ireland), by the colorimetric Reitman Frankel method. A Perlong PU-2018G semi-automated analyser was used to determine the optical density [OD] of the colour change at 546 nm wavelength. The assay protocol was as specified by the Randox ALT and AST Kit manuals.

Determination of Serum Alkaline Phosphatase (ALP)

ALP was assayed with a kit manufactured by Quimical Clinical Aplicada [QCA] Spain. The basic principle is colorimetry. Plasma alkaline phosphatase hydrolyses a colourless substrate of phenolphthalein monophosphate, giving rise to phosphoric acid and phenolphthaledin, which, at alkaline pH, turn into a pink colour that is photometrically determined. A Perlong PUS-2018G semi-automated chemistry analyser was used to measure the optical density [OD] at 550 nm wavelength. The assay procedure is according to the manufacturer's protocol.

Estimation of Total Proteins

Protein concentration was determined by Biuret method. Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of coloured complex (Adekunle et al., 2009).

Estimation of Plasma Albumin

Plasma albumin level was determined by the method described earlier by Oguntibeju et al., (2009). A calibration curve was prepared using various dilutions of the reagent albumin standard, ranging from albumin concentrations of 0 to 50.0 g/L.

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Corresponding concentrations to various optical densities of all the analysed samples were obtained, using the prepared calibration curve and values recorded accordingly in mg/dl.

Histological Studies

Tissues were routinely processed for paraffin wax embedding. Then, 6 µm thick paraffin cross sections containing liver tissues were mounted on slides and stained using routine Haematoxylin and Eosin (H&E) method, and Periodic Acid Schiff (PAS). Protocol for the demonstration of glycogen (Drury and Wallington, 1980) was also followed. The stained sections were examined under a Carl Zeiss research microscope (Axioskope 40, Germany) with a digital camera attached and digital photomicrographs of stained sections were taken.

Statistical Analysis

The data for biochemical parameters were analysed using descriptive and inferential statistics. ANOVA was used to analyse the obtained data, and multiple comparisons were performed using Student's-Newman-Keuls (SNK) test. Primer for windows (McGraw-Hill, version 4.0.0.0) was the statistical package used to analyse data. Results are expressed as means ± standard error of means (SEM). $P<0.05$ was taken as accepted level of significant difference.

Results

In all groups, the animals gained weights during the experimental period (as shown in the Table 1). There was a statistically significant change in mean body weights between Group A rats and the control Group D rats over a period of four weeks. There was no statistically significant difference in the serum levels of the liver enzymes (ALT, AST and ALP) among the groups, although total protein and albumin levels were significantly reduced in Group C animals (as shown in Table 2).

Histological studies showed no cytopathological changes (as seen in Figures 1 and 2), although it is worthy to state that one of the rats in group A showed granuloma, a focal accumulation of activated macrophages, which transforms into epitheloid and giant cells, surrounded by a collar of mononuclear leukocytes, principally lymphocytes, and occasionally, plasma cells (Figure 3).

Table 1: Changes in the body weights of arteether™-treated and control groups of rats over the experimental period of four weeks

Groups	A	B	C	D
Initial weight (g)	158±5.83	170±10.49	168±9.17	168±9.17
Final weight (g)	226±8.72	228±13.56	230±12.25	220±8.94
Weight gain (g)	68±3.74*	58±3.74	62±3.74	52±2.

Values are given as means ± SEM for five rats in each group. * denotes P<0.05 compared to control (n=5).

Table 2: Effects of arteether™ on selected biochemical parameters

Groups	A	B	C	D
Alanine Aminotransferase (ALT) (iu/L)	73.2±19.37	72.6±20.5	100±6.69	112.8±10.33
Aspartate Aminotransferase (AST) (iu/L)	88.4±27.4	91.6±30.55	133±14.93	149±14.06
Alkaline Phosphatase (ALP) (iu/L)	64.9±22.67	52.3±19.52	72.8±14.72	57.0±22.37
Albumin (mg/dl)	4.5±0.24	4.82±0.10	3.88±0.13*	5.22±0.28
Total Protein (TP) (mg/dl)	8.58±0.37	9.18±0.42	7.72±0.50*	9.84±0.63

Values are given as means ± SEM for five rats in each group. * denotes P<0.05 compared to control (n=5)

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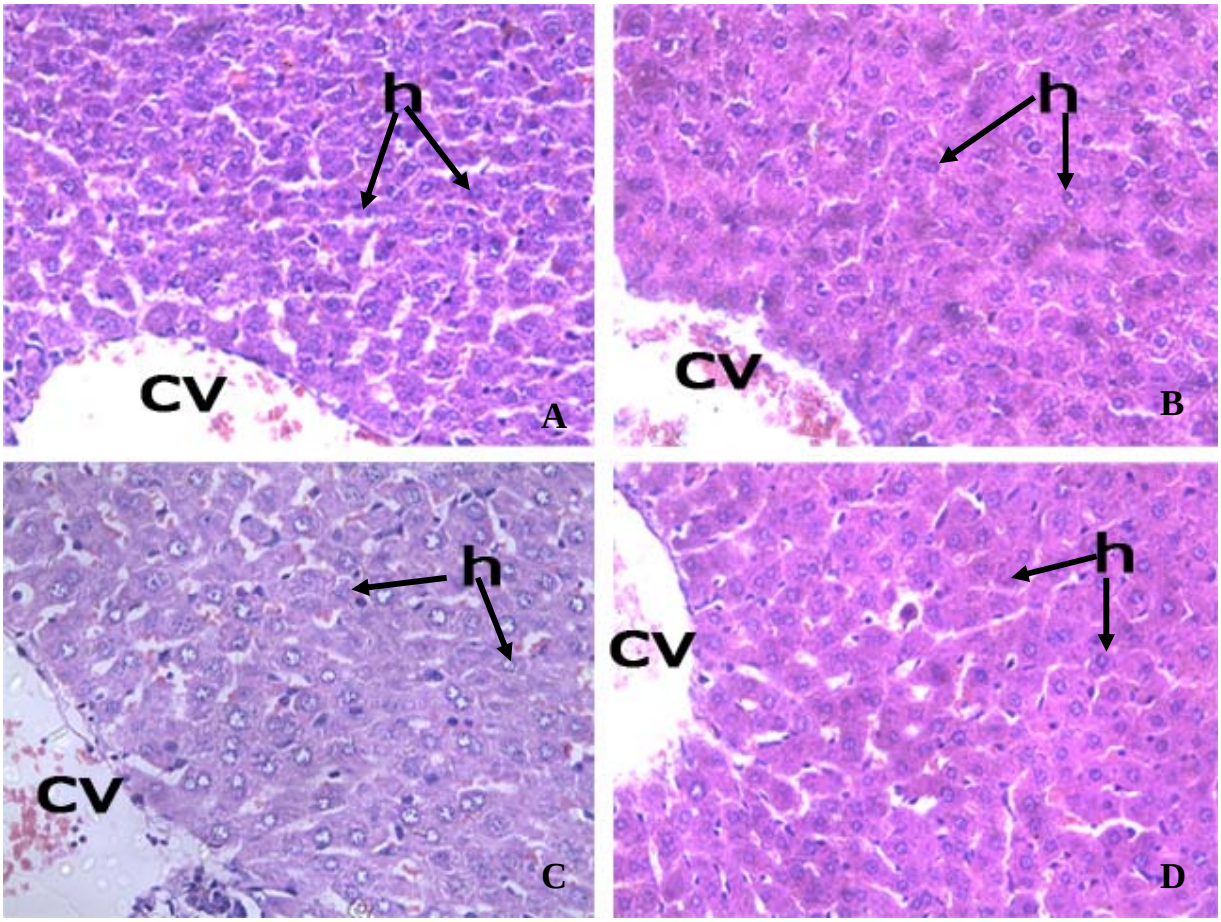

Figure 1: Light photomicrographs of the livers of Groups A, B, C and D rats. H&E x400. No cytopathological changes were noted. Central Venules (CV), Hepatocytes (H)

Discussion

Arteether™ is a useful drug against chloroquine-resistant *Plasmodium falciparum* malaria, without an increased incidence of toxicity. It has a longer half-life, and has more lipophilic properties than artemether™, which aids its accumulation in brain tissues for the treatment of cerebral malaria. Other advantages of arteether™ include its stability, compared with sodium artesunate. Its biochemical breakdown does not give methanol, as does artemether™; and it is easily formulated in oil for parenteral administration (Valecha and Tripath, 1997). However, tolerability of arteether™ is similar to that of other artemisinin derivatives. Arteether™ also has the additional advantage of requiring only a three-day regimen, compared with other artemisinin derivatives, which require a seven-day regimen as monotherapy (Anil et al., 2006). Pharmacodynamic studies on arteether™ have shown that both ß-arteether™ and α/ß-arteether™ have comparable activity (Anil et al., 2006). However, their modes of action, which include release of free radicals, have the potential to cause cytotoxicity in various organs of the body (Chris, 2004).

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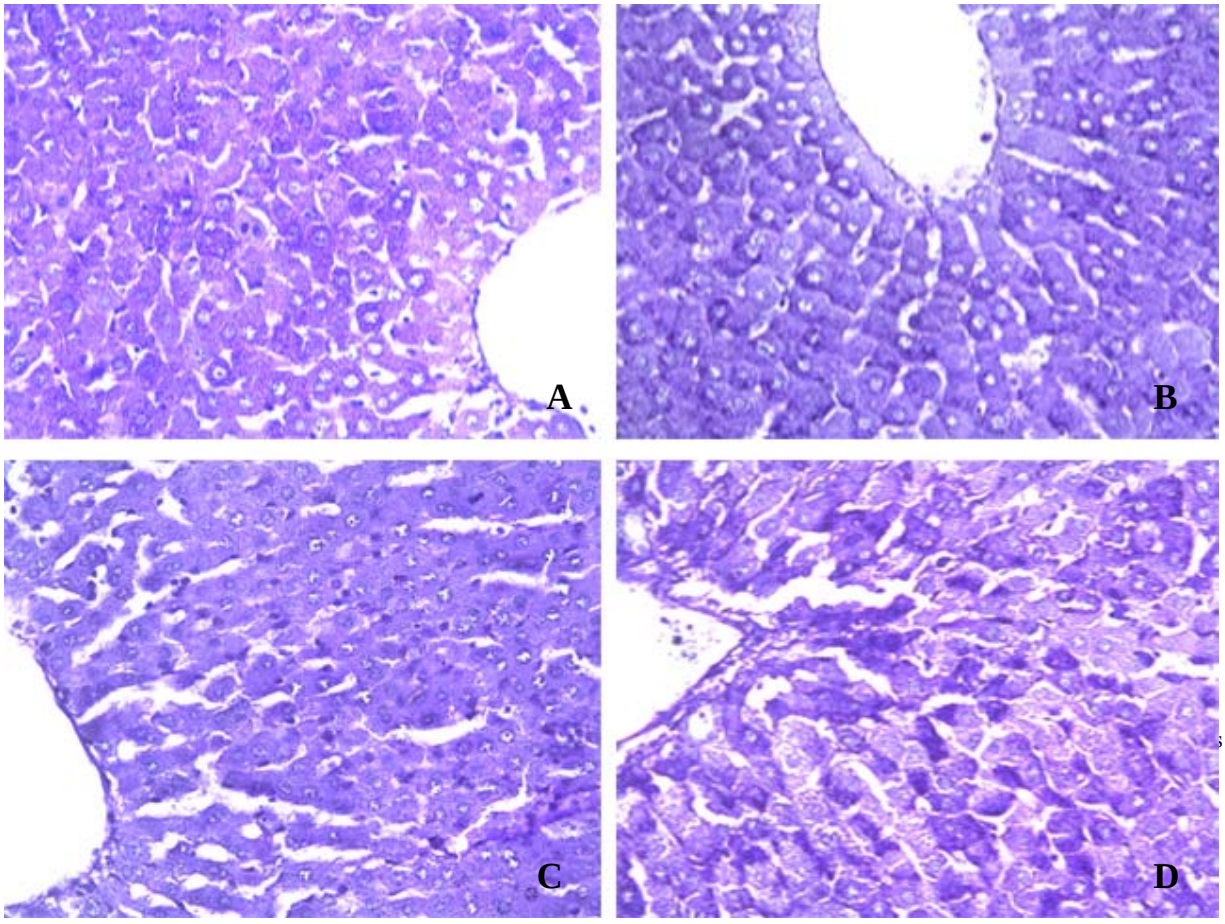

Figure 2: Light photomicrographs of the livers of Groups A, B, C and D rats. PAS x400; Periodic acid-Schiff reaction shows distribution of glycogen (darker colour) in the hepatocytes

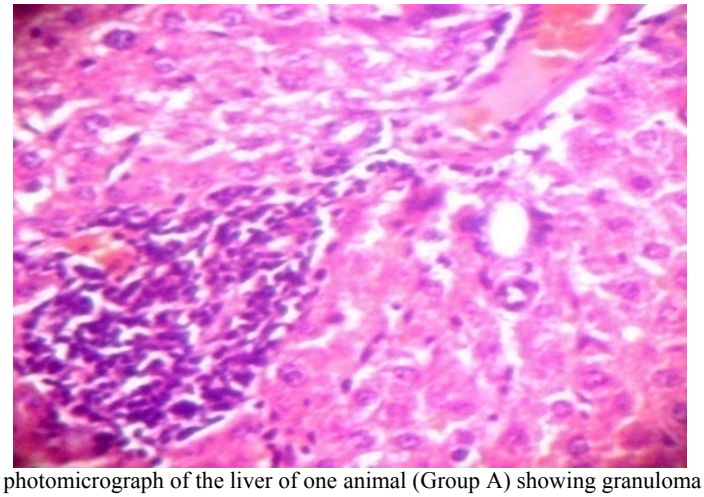

Figure 3: Light photomicrograph of the liver of one animal (Group A) showing granuloma (**G**). H&E X400

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All artemisinin-derived agents are metabolised in the liver to their active metabolite, dihydroartemisinin, which makes hepatocytes vulnerable to the adverse effects of this group of drugs.

In this study, all the animal groups gained weights over our four-week study period. The only statistically significant difference in weight gain was noted between Group A rats which received standard dose of arteether™ (3mg/kg daily for three days) and Group D rats, the control group ($p<0.05$). It is most likely that arteether™ stimulates appetite at normal, therapeutic doses, which may be counter productive at higher doses, or close repeated treatment regimens. The limitation of this observation is that attention was not paid to differences in water and food consumption of the animals during our experiment.

There was no statistically significant difference in liver enzyme (alanine transaminase, aspartate transaminase, and alkaline phosphatase) levels of the animals treated with arteether™ and the control group. Adekunle et al., (2009) reported a transient decrease in serum concentrations of total protein and albumin in malaria patients treated with artemether™, suggesting that the drug may have effect on the liver, and that the altered circulating concentration of albumin in the human study may be due to side-effects of artemether™ on liver cells (Grcae et al., 1997). Proteins are synthesised by the liver. However, the protein synthetic capability of the liver may be affected by any chemical that may have either moderate or extensive hepatotoxic effects. Most drugs bind to albumin and globulins. However, it is usually the free fraction of drugs that elicits the desirable therapeutic effects observed during drug treatment. Any condition that may reduce the circulating concentrations of proteins, particularly albumin, will alter the binding capability of a drug. This can thereby lead to a high concentration of the free fraction, which may be higher than required by the body, thus making the drug to exert undesirable effects (Adekunle et al., 2009). Therefore, the significant decrease in both total protein and albumin in the present study, even though liver enzymes were not significantly influenced, may suggest a mild adverse effect of arteether™, following prolonged usage.

Histological review of the livers of arteether™-treated Wistar rats in this study revealed normal cyto-architecture similar in all animals, except for one animal in Group A which showed focal granuloma. Cases of significant liver inflammation has also been reported in association with prolonged use of relatively high-dose artemisinin (Leonardi et al., 2001). This finding may indicate an idiosyncratic, allergic type-B drug reaction.

The present findings suggest that the metabolism of arteether™ does not cause significant hepatocellular damage in Wistar rats at therapeutic doses. The various adverse reactions attributed to arteether™ may be multifactorial, and may include (a) interaction between arteether™ and other drugs, (b) co-morbidity, and/or (c) malaria itself. In conclusion, this study shows that arteether™ is well tolerated in Wistar rats at therapeutic doses. Further studies with higher concentrations of the drug, and more extensive review of enzyme biomarkers of the liver are, however, necessary to establish the safety of arteether™.

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