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Full Length Research Paper

An evaluation of the effects of pre-treatment with vitamin C and folic acid on the hepatotoxic effect of artemisinin combination

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Artemisinin and its derivatives are among the most effective antimalarial drugs used today. This study compared the effects of pre-treatment with vitamin C and folic acid on the hepatotoxic effect of artemisinin combination. Thirty male wistar rats randomly assigned to six groups of five rats each were used in the study. Group A was the normal control; groups B, C, D, E and F were placed on therapeutic doses of leonart, leonart+vitamin C, leonart + folic acid, vitamin C and folic acid respectively. They were pretreated with the respective supplements for 28 days while administration of leonart was carried out for four days starting from the 24th day of supplements administration. The rats were thereafter sacrificed by cervical dislocation after an overnight fasting. Serum prepared from the whole blood and homogenized liver were then used for analysis. The result indicates that leonart administration raised the level of malaondialdehyde, serum total, conjugated bilirubin and the activities of alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). It however decreases the activities of liver catalase, superoxide dismutase (SOD) and glutathione (GSH) level. Combined administration of leonart with vitamin C restored these parameters to pretreatment levels. Neither separate nor combined administration of folic acid with leonart altered any of these parameters.

Key words: Leonart, hepatotoxicity, artemisinin, antimalarial, oxidative stress.

INTRODUCTION

Malaria is considered to be a major public health problem in Nigeria. It causes more than 50% of the disease burden (Federal MoH, 2005) and almost 50% of all-cause health expenditure (Onwujekwe, et al., 2000). 20% of all hospital admissions, 30% of outpatient visits, and 10% of hospital deaths are attributable to malaria, and half of Nigeria's population is exposed to at least one episode of malaria every year (Okeke et al., 2006). Results of a

modelling exercise presented in the National Malaria Control Program (NMCP) strategic plan 2009-2013 show that malaria accounts for an estimated 300,000 deaths in children under five each year and 11% of the maternal mortality burden in Nigeria. Malaria is responsible for 25% of all infant-related mortality and 30% of child-related mortality (ACT watch, 2009). In relative terms, Nigeria contributes more than a third of the total African malaria burden (RBM, 2008).

Malaria is a mosquito-borne infectious disease of humans and other animals caused by *Plasmodia* and are also definitely the single most destructive and dangerous infectious agent and the most serious health challenge in the developing countries of the world (Christopher et al., 2012). There were estimated 225 million cases of malaria worldwide in 2009. In 2010, there were 219 million malaria cases leading to approximately 660,000 malaria deaths, mostly among African children (UNICEF 2013).

Currently, artemisinin-based combination therapy (ACT) is recommended for the treatment of *P. falciparum* malaria (Singh et al., 2004; WHO, 2011). In the treatment, fast acting artemisinin based compounds are combined with a drug from a different class. The artemisinin-derivatives, artemether, artesunate, and dihydroartemisinin, are currently the most potent anti-malarial medicines on the market. They are widely available in the different pharmaceutical dosage forms including tablets, injections, suppositories and dry powders. Leonart (an artemeter-lumefantrine) was introduced as alternative drugs for the treatment of malaria in Africa due to the emergence of drug-resistant *Plasmodium falciparum*. The drug is effective in the treatment of malaria and, in artemisinin-based combination chemotherapy with other effective blood schinzonticide to prevent recrudescence and delay the selection of resistant strains. However, it is not recommended for the treatment of malaria caused by *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae*, because other effective antimalarials are available. It is a fixed-dose combination of artemether and lumefantrine. It is used in the treatment of uncomplicated malaria caused by pure or mixed *P. falciparum* infections including strains from multidrug resistant areas and can prevent recrudescence after artemether therapy. Previous studies had reported that oral administration of artemisini-lumefantrine combination increases liver oxidative stress and caused significant elevation of serum alanine transaminase (ALT) and aspartate aminotransferase (AST) (Adaramoye et al., 2008).

Although medical doctors now prescribe the highly effective Artemisinin-based Combination Therapies (ACT) for the treatment of malaria, some study has however warned that taking ACT in combination with multivitamins that contain iron and vitamin C and E renders the drug ineffective (Looareesuwan et al., 1998; Patricia et al., 2009; Adumanya et al., 2012). The most prominent role of vitamin C is its immune stimulating effect, which is important for the defense against infections such as common colds. It also acts as an inhibitor of histamine, a compound that is released during allergic reactions. As a powerful antioxidant, it can neutralize harmful free radicals and aids in neutralizing pollutants and toxins. It is also able to regenerate other antioxidants such as vitamin E. Thus it is able to prevent the formation of potentially carcinogenic nitrosamines in the stomach (due to consumption of nitrite-containing foods, such as smoked meat) (Adunmaya et al., 2012). It has been reported that the concomitant administration of Artemisinin in various forms (Artemether, Arteether, Artesunate, Artenilate) with vitamin C and multivitamins - especially those preparations containing trace elements and minerals (such as Zinc, Copper, Iron (Fe^{2+}), etc) with antioxidant properties reduces the efficacy of the Artemisinin-based antimalarial drug therapy (George and Nmoka, 2003). However, there is paucity of information regarding the role of this vitamin in hepatic injury associated with ACT therapy. It is therefore, the purpose of this work to examine the protective role of vitamins C and folic acid on artemisinin-lumefantrine induced hepatotoxicity in rats.

MATERIALS AND METHODS

Drug

Leonart® composed of 80 mg Artemether and 480 mg lumefantrine was manufactured by Bliss GVS Pharma Ltd. India. And obtained from Fontana Pharmacy, Ijebu-Ode, Ogun State, Nigeria.

Experimental design

Thirty male adult rats of the Wistar Kyoto strain weighing 100 to 120 g obtained from the Animal house of the Department of Physiology, University of Ibadan, Nigeria were used for the study. All the animals were housed in metallic cages and maintained in well ventilated room provided with 12:12 h light and dark cycle for each 24 h period at a temperature of approximately 25°C. They were fed on pellets and tap water ad libitum. After the initial period of 7 days acclimatization, the animals were randomly assigned into 6 groups of 5 rats per group labeled as:

Group A (Normal control): Administered with normal saline
Group B (Test control 1): Administered with leonart
Group C (Test 1): Pre-treated with vitamin C thrice daily for 24 days and then administered with ACT combination therapy.
Group D: (Test 2): Pre-treated with folic acid for 24 days and then administered with leonart combination therapy.
Group E (Test control 2): Administered with vitamin C.
Group F (Test control 3): Administered with folic acid.

Drug administration

Leonart was administered at a therapeutic dose of 2.67 mg artemether/16 mg lumefantrine per Kg body weight twice daily. Vitamin C was administered at a dose of 53.71 mg/kg thrice daily

Table 1. Effect of pre-treatment with vitamin C and folic acid on leonart induced oxidative stress.

Group/Treatment	MDA (µmol/mg)	Catalase activity µmol/mg protein	SOD activity µmol/mg protein	GSH (µg/g$_3$tissue) x10
Group A (Normal saline)	1.49 ± 0.04^a	170.33±1.56^a	0.55±0.40^a	17.02±0.05^a
Group B: (Leonart)	2.14±0.10^c	161.67±2.63^c	0.42±0.35a	12.14±0.03^c
Group C (Leonart + Vitamin C)	1.51 ± 0.07^a	196.67±1.8	0.72±0.24^a	15.63±0.51^a
Group D (Leonart + folic acid)	1.66±0.06^b	87.24±6.91^c	0.58± 0.06^a	16.01±0.05^a
Group E (Vitamin C)	1.41 ± 0.04^a	159.67±6.06^a	0.63±0.43^a	16.09±0.06^a
Group F (Folic acid)	1.46 ±0.02^a	149.04± 8.61^a	0.67± 0.05^a	16.61±0.12^a

Results are mean ± SEM of 5 determinations, values in the same column with similar superscripts are not significantly different from each other.

while folic acid was administered at a dose of 50 mg/kg. Groups C, D, E and F were treated with the respective supplement for 28 days. On the 24th day after the commencement of treatment, leonart administration was carried out in the rat groups B, C and D. The administration was done for the next four days at the respective dosage.

Preparation of tissue homogenate

Rats were sacrificed 24 h after the last administration of leonart and or supplements after an overnight fast. Liver were quickly removed and washed in ice-cold 1.15% KCl solution, dried and weighed. The liver samples were homogenized in 4 volumes of 5 mM phosphate buffer, pH 7.4 and centrifuged at 10,000 ×g for 15 min to obtain post-mitochondrial supernatant fraction. The samples were stored at –80°C until use. All procedures were carried out at temperature 0 to 4°C.

Preparation of serum

Blood was collected from the inferior *vena cava* of heart of the animals into plain centrifuge tubes and was allowed to stand for 1 h. Serum was prepared by centrifugation at 3000 × g for 15 min in a centrifuge. The clear supernatant was used for analysis.

Assay procedure

Protein determination

Serum and liver protein levels were determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

Total and conjugated bilirubin concentration

The bilirubin levels (total and direct) were assayed for by the method of Rutkowski and Debaare (1966). The method is based on the reaction between bilirubin and diazotized sulfanilic acid in alkaline medium to form a blue-coloured complex, which was read spectrophotometrically at 546 nm.

Liver dysfunction marker enzymes

Serum alanine aminotransferase (ALT) and aspartate aminotransferases (AST) activities were determined using a combination of the methods of Mohun and Cook (1957), and Reitman and Frankel (1957). The estimation of alkaline phosphatase (ALP) activities was based on the method of Williamson (1972). ALP activity was measured spectrophotometrically by monitoring the concentration of *p*-nitrophenol formed when ALP reacts with *p*-nitrophenyl phosphate at 405 nm.

Liver oxidative stress

The extent of lipid peroxidation (LPO) was estimated by the method of Buege and Aust (1978). The method involved the reaction between malondialdehyde (MDA; product of LPO) and thiobarbituric acid to form a pink precipitate, which was read at 535 nm spectrophotometrically. Superoxide dismutase (SOD) activity was measured by the nitroblue tetrazolium reduction method of McCord and Fridovich (1969); Catalase (CAT) activity was assayed by measuring the rate of decomposition of hydrogen peroxide at 240 nm as described by Aebi (1974); reduced GSH level was assayed by measuring the rate of formation of chromphoric product in a reaction between DTNB (5,5′ -dithio-bis (2-nitrobenzoic acid) and free sulfhydryl groups (such as reduced glutathione) at 412 nm according to the method of Moron et al. (1979).

Statistical analysis

All values were expressed as mean ± S.D. The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) (Duncan, 1957). Test with P< 0.05 were considered significantly different.

RESULTS

Leonart was observed in the study to increase the MDA value significantly above the normal control value and also reduced the catalase activity but does not significantly altered the liver SOD activity significantly (Table 1). Combined administration of vitamin C and leonart lowers the level of peroxidation to pretreatment value and also increased the catalase activity above the normal control level but does not significantly altered the SOD activity. Table 1 also indicates that whereas separate administration of folic acid neither altered the liver MDA level nor the antioxidant enzyme activities, its combined administration with leonart raised the level of peroxidation above the normal control value (though the value obtained was lower than that which was observed

Table 2. Effect of combined administration of supplements and leonart on some liver enzymes.

Group/Treatment	Liver enzyme activity (U/L)		
Group/ Treatment	**ALP**	**ALT**	**AST**
Group A (Normal saline)	84.57±12.85[a]	24.33±1.23[a]	52.33±8.69[a]
Group B: (Leonart)	143.00±1.84[c]	36.00±2.89[b]	71.83±1.78[c]
Group C (Leonart + vitamin C)	106.67±4.21[b]	25.66±1.74[a]	66.17±2.22[b]
Group D (Leonart + folic acid)	113.85±2.59[b]	30.00 5.07[a]	52.00 3.14[a]
Group D (Vitamin C)	91.65±2.48[a]	20.17±1.34[a]	44.00±1.06[a]
Group F (folic acid)	81.75± 2.45[a]	20.03± 1.84[a]	55.00± 3.18[a]

Results are mean ± SEM of 5 determinations, values in the same column with similar superscripts are not significantly different from each other.

Table 3. Effect of combined administration of supplements and leonart on serum bilirubin and total protein.

Group/ Treatment	Total bilirubin (mg/dL)	Conjugated bilirubin (mg/dL)	Total protein (mg/dL)
Group A (Normal saline)	4.60±0.06[a]	3.00±0.05[a]	105.10±5.63[a]
Group B: (Leonart)	7.60±0.07[c]	4.30±0.01[b]	90.08±5.77[b]
Group C (Leonart + vitamin C)	4.80±0.04[a]	3.02±0.03[a]	100.06±5.92[a]
Group D (Leonart + folic acid)	6.89±0.13[b]	4.10±0.01[b]	112.11±3.18[a]
Group E (Vitamin C)	4.00±0.06[a]	3.20±0.04[a]	102.06±3.16[a]
Group F (folic acid)	4.00±0.06[a]	3.10±0.05[a]	105.24±3.12[a]

Results are mean ± SEM of 5 determinations, values in the same column with similar superscripts are not significantly different from each other.

when leonart was administered alone). No significant alteration was found in the liver catalase and SOD activities when folic acid was administered separately (when compared with that of the normal control). Combined administration of leonart and folic acid however significantly lowered the liver catalase activity. The observed activity was also lowered than that of the normal control value and that obtained with vitamin C administration and when vitamin C was combined with leonart. The result of the liver GSH analysis also indicates that leonart administration significantly reduced the liver GSH below the normal control value. When leonart was co-administered with vitamin C, the liver GSH was brought to the pre treatment level. Neither separate treatment with folic acid and vitamin C nor combined administration of folic acid with leonart was observed to have altered the liver GSH level.

The result also shows that separate administration of leonart increased the serum ALP, ALT and AST activities above the normal control values. When leonart administration was combined with vitamin C, the serum ALP and AST activities were significantly reduced below that obtained with separate administration of leonart but the observed activities were also higher than the normal control values. The liver enzyme activities were not significantly altered with separate administration of folic acid. The ALP activity was however raised above the normal control value when folic acid administration was combined with leonart. When the enzyme activities were compared when folic acid and vitamin C were separately administered, they were not observed in this study to be significantly different (Table 2).

Result of the serum bilirubin shown in Table 3 indicates that administration of leonart significantly raised serum total and conjugated bilirubin significantly above the normal control values. When leonart was co-administered with vitamin C, the observed total and conjugated bilirubin values were not different from that of the normal control values. Co-administration of leonart and folic acid however raised the total and conjugated bilirubin level above the normal control values. The observed values were also higher than that obtained when folic acid was separately administered. The table also indicates that leonart administration significantly lowered serum total protein level below the normal control value. The serum total protein however does not vary significantly when leonart was co-administered with either vitamin C or folic acid neither was it altered with separate administration of vitamin C and folic acid.

DISCUSSION

The increased oxidative stress observed in this study which is evident with increased liver MDA level and reduced liver antioxidant status with leonart administration is a confirmation of previous reports on the oxidative stress associated with artemisinin combination

therapy (Adaramoye et al., 2008; Paul et al., 2010; Akomolafe et al., 2011) . The significant increase in MDA concentration observed in leonart administered rats is an indication of the tendencies of leonart to predispose to oxidative stress. Artemisinin in its chemical structure contains a fragile di-oxygen (O---O) bridge and its antiplasmodial action is predicated on the generation of an oxygen centered free radical due to transient cleavage of this bridge which thus results in lipoprotein oxidation, endothelial dysfunction and vascular damaging. The uptake of oxidized LDL particles by scavenger receptors present in macrophages triggers a series of events, leading to production of foam cells and therefore the formation of atherosclerotic plaque (Nwanjo et al., 2007). Vitamin C has been shown in our study to reduce the oxidative stress induced by leonart administration. This is evident by the reduction in liver MDA and increased liver GSH and catalase activity when vitamin C was co-administered with leonart. The mechanism by which this was achieved may be due to the antioxidant property of vitamin C. Vitamin C, being antioxidant will mop up free radical generated by the transient cleavage of the endoperoxide bridge in the artemisinin structure thereby reducing the level of liver peroxidation. It may however, also be important to point out here that the antimalarial activity of leonart is a function of the oxidation effect of the drug. An antioxidant such as vitamin C therefore that does mop up the free radicals when co-administered with artemisinin combination drug such as leonart, although may be beneficial in reducing liver oxidative stress (as evident in our study) but may also incapacitate the drug molecule and may reduce the effectiveness of its anti protozoal action as it may increase plasma concentrations of the drug and delays reaching peak drug concentration (Owira and Ojewole, 2010). This may thus increase the risk of serious malarial problems.

Folic acid administration in this study was not seeing to have either reduce or increase the level of oxidative stress that was associated with leonart administration. Folic acid (folate) is a water-soluble B-vitamin whose biologically active form is tetrahydrofolic acid (THF), which participates in the transfer of 1-carbon units (such as methyl, methylene, and formyl groups) to the essential substrates involved in the synthesis of DNA, RNA, and proteins (Bailey and Gregory, 1999). Ingested folic acid can be converted to its physiological forms. This process is initiated by dihydrofolate reductase in a two-step reaction; the first step being the conversion to dihydrofolate (DHF). This is a slow and rate-limiting step (Wagner, 1995). In the second, more rapid step, dihydrofolate is further reduced to THF. THF can then be converted into additional physiological folates including 5-methyl-THF, the form that is found in circulation and in tissues (Parcia et al., 2008). Folic acid especially at high doses has been reported to counteract the efficacy of antimalarial drug sulfadoxine-pyrimethamine (UNICEF, 2013). Our study does not suggest a similar effect with leonart.

The liver plays a central role in transforming and clearing chemicals and is susceptible to toxicity from these agents. This is primarily because of its unique metabolic responsibility and close relationship with the GIT. Consequently, certain medicinal agents when taken in overdose and sometimes even when introduced in therapeutic ranges may injure the organ. Liver enzymes are usually raised in acute hepatotoxicity, but tend to decrease with prolonged intoxication due to damage to the liver (Obi et al., 2004). Previous studies have reported that some antimalarial agents such as chloroquine (Pari and Amali, 2005) and amodiaquine (Farombi et al., 2000) can induce hepatic damage. The pharmacokinetics of ACTs shows that their primary site of metabolism is the liver, thus it would be expected that the liver would be susceptible to injury from these agents. Previous authors however, did not agree on the capacity of ACT to induce liver injuries. Whereas Adaramoye et al. (2008) reported increased liver damage in rats administered with ACT, Georgewill and Ebong (2012) reported a normal hepatic cells in mice administered with ACT. In the present study, we observed increased serum ALT, ALP and AST activities in rats treated with therapeutic dose of leonart. This may suggest the ability of the drug to predispose to hepatic injury. Furthermore, our study indicates that co-administration of vitamin C with artemisinin may prevent this hepatic injury predisposition. Bilirubin is a tetrapyrrole created by the normal breakdown of heme. Most bilirubin is produced during the breakdown of hemoglobin and other hemoproteins. Accumulation of bilirubin or its conjugates in body tissues produces jaundice which is characterized by high plasma bilirubin levels and deposition of yellow bilirubin pigments in skin, sclerae, mucous membranes, and other less visible tissues (Tiribeli, 2005; Drummond and Kappas, 2004). An elevated level of bilirubin in the blood may indicate liver disease or drug-induced liver damage. Elevations of direct bilirubin typically result from obstruction either within the liver (intrahepatic) or a source outside the liver (e.g. gallstones or a tumor blocking the bile ducts). Bilirubin measurements are especially valuable in newborns, as extremely elevated levels of unconjugated bilirubin can accumulate in the brain, causing irreparable damage.

The result of alteration in both total and conjugated bilirubin level in leonart administered rats' correlates with that of variation in liver enzymes which also suggest the ability of the therapeutic administration of the drug to induce liver injury and the efficacy of vitamin C to prevent this damage. The study thus suggests that the vitamin offer protection by preserving the structural integrity of hepatocellular membrane against ACT injury. The ability of the vitamin to offer this protection could also be attributed to its antioxidative effect which was also confirmed in our study. This finding correlates with previous study, which reported that treatment with

tetrahydrocurcumin (an antioxidative substance derived from curcumin, the component of turmeric) significantly reduced the activities of serum hepatoxicity markers in chloroquine-induced hepatoxicity (Pari and Amali, 2005) and erythromycin-induced hepatoxicity (Pari, 2004). We however do not observe the same protection with folic acid administration.

Conclusion

The result of our study correlates' with previous findings that therapeutic dose of artemisinin combination therapy may induce hepatotoxicity. This study also indicates that co-administration of ACT with vitamin C may offer hepatotoxic protection but that co-administration of the drug with folic acid may not. We however, suggest caution in administering vitamin C along with ACT since the antioxidant activities of the vitamin may mop up the free radicals generated by the cleavage of the di oxygen bridge of the drug and this may reduce the efficacy of the drug against the malarial plasmodium.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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