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EFFECT OF ARTESUNATE ON LIVER FUNCTIONS OF THE WISTER RAT

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ABSTRACT: *Antimalarial drug toxicity is viewed different, depending on if the clinical indication is for treatment or prophylaxis. In drug therapy of Plasmodium falciparum malaria, which has a high mortality if untreated, a greater risk of adverse reactions to antimalarial medication is inevitable. The effect of the administration of Artesunate on the liver of wistar rats was studied. Study design was experimental and deployed clinical laboratory assessments. Four groups of wistar rats, each of five animals weighing between 100-150 g were used. Group 1 served as the control and was administered normal feed and drinking water. Group 2, 3 and 4 received 0.24mg/kg, 0.34mg/kg and 76mg/kg body weight Artesunate daily respectively, orally for four weeks. Serum Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP) activities and Bilirubin were determined at the end of the treatment. Results showed that in group 3 and 4, there was a significant increase in serum AST and ALT and a significant decrease in serum ALP. The results also showed that at mild doses (0.24mg/kg and 0.34mg/kg), Artesunate promoted weight gain and at highest dose (76mg/kg), it appeared to result in reduced percentage weight gain suggesting perhaps that high doses were toxic. It is concluded, that administration of high doses of Artesunate by the oral route produced considerable damage to the liver.*

KEYWORDS: Malaria, Artesunate, Serum, Enzymes, Wister Rat

INTRODUCTION

Antimalarial medicines prevent or treat malaria, a febrile illness that threatens approximately 3.4 billion people living in 106 countries/territories, and is responsible for 500,000 deaths in 2013[1]. Malaria is transmitted to humans (hosts) through the bites of infected mosquitoes (vectors) and the plasmodium parasite, a member of the one-celled protozoa is the causative agent[2].

The artemisinin antimalarials are several sesquiterpene lactone compounds (Artesunate, Arteether, Dihydroatemisinin and Artelinic acid) synthesized from the plant Artemisia annua. These compounds are used for treatment of severe malaria with very rapid clearance of all asexual stages of Plasmodium falciparum and faster fever resolution than occur with quinine [3][4].

In recent years, artemisinin use has grown as parasite strains became resistant to antimalarial drug classes, particularly the Ary1 amino alcohol compounds such as chloroquine. [5]. Artemisinin-based Combination Treatments (ACT) stand approved by the World Health Organization (WHO) as first-line treatment for uncomplicated falciparum malaria[6][5].

Hence, combination therapy (CT) which is based on concurrent use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite is now standard[7]. Accordingly, oral fixed combinations of artesunate with a long half-life drug like mefloquine, amodiaquine and lumefantrine (half-life of about 3 to 6 days) are licensed and available in Nigeria.

Pharmacokinetics

Artesunate is a commonly used water-soluble hemisuccinate derivative of artemisinin. It can be administered through enteral (oral and rectal) and parenteral routes (intramuscular and intravenous). The oral formulation is probably completely hydrolysed before entering the systemic circulation. For severe malaria, parenteral administration is advocated. Artesunate is unstable in neutral solution and the injectable formulation must be prepared immediately before use in 5% (w/v) sodium bicarbonate solution to produce the salt sodium artesunate.

Artesunate is rapidly hydrolysed to its active metabolite Dihydroartemesinin (DHA). DHA substantially accumulates in P. falciparum-infected red blood cells although artesunate itself is not significantly protein-bound. Artesunate has a plasma half-life of 3-29 minutes while DHA has a plasma half-life of 40 to 95 minutes. The modes of excretion of DHA have not been fully elucidated.

Pharmacodynamics

There is no consensus mechanism of action of artemisinin, but two major pathways are elucidated, an iron (or haem) cleavage [8][9] and the action on calcium transporters PfATP618[10].

When red blood cells are infected, the parasite catabolizes readily available hemoglobin and liberates haem from an iron-porphyrin complex. The free ferrous ion (Fe^{2+}) generates highly reactive free radicals (reactive oxygen species) that cleaves the endoperoxide bridge [11], which damages the parasite's DNA[12].

DHA binds tightly to parasite infected red blood cell membrane and inhibits the P. falciparum encoded sarcoplasmic/endoplasmic reticulum calcium ATPase [13]. The likely inhibitory target is PfATP6, a SERCA-type enzyme (calcium transporters). Artemisinin has also been posited to compete with thapsigargin for SERCA binding, though artemisinin is much less toxic to mammalian cells[14]

Aim and Objectives

To determine the effect of artesunate on some biochemical parameters Alanine Aminotransferase (ALT), Aspartate Aminotransferase, Alkaline phosphatase (ALP) and bilirubin levels associated with the liver functions in Wister rat.

MATERIALS AND METHODS

Materials

Kits: Randox kits (Randox laboratories Ltd, Ardmore, Diamond Road, crumbling, co. Antrim, United Kingdom, and BT294 QY) were purchased and used for the enzyme assays.

Animals: Twenty (20) white albino rats weighing between 100-150g were purchased from the animal house unit, National Veterinary Research Institute (NVRI) Vom, Plateau state, Nigeria.

List of Major Equipment

- Water bath (thermoshaake, S.W.B 25)
- Weighing balance (Ohau's, cooperation, USA)
- Conical flask
- Spectrophotometer (Ryan, Science and Instrument Company England).
- Mechanical Shaker
- Bench Centrifuge

List of Chemicals

- 2,4-dinitropheny1hydrazine
- Sulphanilic acid
- Hydrochloric acid
- Sodium nitrate caffeine
- Sodium benzoate
- Nak – Tatrate
- Sodium hydroxide pellets

METHODS

Study was an experimental design, using clinical laboratory assessments. The albino rats were placed into 4 groups (ABC and D) of 5 rats each. The animals were housed in plastic cages at room temperature and fed with a commercial diet (Vital Feed: Grand Cereals and Oil Milled, Jos).

The animals in group A served as the control group (normal feed and water). Group B C and D were given different doses of the antimalarial drug by gavage for 5 days.

After 5 days artesunate administration, the rats were sacrificed, and the blood drained, allowed to clot and then centrifuged to obtain serum. The levels of aspartate aminotransferase (AST), alkaline aminotransferase (ALT), alkaline phosphatase (ALP) enzyme activities and bilirubin levels were determined using the kits.

Test for Glutamate Oxaloacetate Transaminase (GOT)

Principle:

$$\text{a-oxoglutarate + L-Aspartate} \xrightarrow{GOT} \text{L-glutamate + Oxaloacetate}$$

Glutamic-Oxaloacetate Transaminase was measured by monitoring the concentration of Oxaloacetate hydrozone formed with 2,4-dinitropheny1-hydrazine.

Sample Material: Serum

REAGENT COMPOSITION

Contents	Initial concentration of solutions
1. Buffer	
• Phosphate buffer	100mmo1/1, pH 7.4
• L-Aspartate	100mmo1/1
• a-oxoglutarate	2mmo1/1
2. 2,4-dinitrophenyldrazine 2mmo1/1	2mmo1/1

Procedure:
Wavelength: Hg 546nm
Cuvette: 1cm light path
Incubation Temperature 37˚C
Measurement against reagent blank
Pipette into test tubes

Reagent	Blank	Sample
Sample	--------	0.1 m1
Solution 1	0.5m1	0.5m1
Distilled water	0.1m1	------

Mix and incubate for exactly 30 minutes at 37˚C

Solution 2	0.5m1	0.5m1

Mix and allow to stand for exactly 20 minutes at 20 to 25˚C

Sodium hydroxide	5.0m1	5.0m1

Mix, read the absorbance of sample (A sample) against the reagent blank after 5 minutes
.

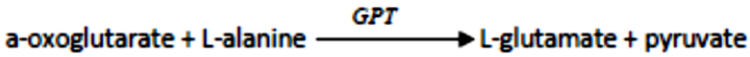
Calculation:

Table1: Obtaining the activity of GOT in the serum from the table

Absorbance	**U/1**	**Absorbance**	**U/1**
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

Test for Glutamic-Pyruvic Transaminase (GPT)

Principle:



Glutamic-Pyruvic Transaminase was measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenyIhydrazine.

Contents	Initial concentration of solutions
1. Buffer	
• Phosphate buffer	100mmo1/1, pH 7.4
• L-Alanine	200mmo1/1
• a-oxoglutarate	2.0mmo1/1
2. 2,4-dinitrophenyldrazine	2.0mmo1/1

PROCEDURE:

Wavelength:	Hg 546nm(530 – 550nm)	
Cuvette:	1cm light path	
Incubation Temperature	37˚C	
Measurement against reagent blank		
Pipette into test tubes		
Reagent	Blank	Sample
Sample	--------	0.1 m1
Solution 1	0.5m1	0.5m1
Distilled water	0.5m1	------
Mix and incubate for exactly 30 minutes at 37˚C		
Solution 2	0.5m1	0.5m1
Mix and allow to stand for exactly 20 minutes at 20 to 25˚C		
Sodium hydroxide	5.0m1	5.0m1

Mix, read the absorbance of sample (A sample) against the reagent blank after 5 minutes

Calculation:

Table 2: Obtaining the activity of GPT in the serum from the table

Absorbance	u/1	Absorbance	u/1
0.025	4	0.0275	48
0.050	8	0.0300	52
0.075	12	0.0325	57
0.0100	17	0.0350	62
0.0125	21	0.0375	67
0.0150	25	0.400	72
0.0175	29	0.425	77

0.0200	34	0.450	83
0.0225	39	0.475	88
0.250	43	0.500	94

Test for Alkaline Phosphatase

Principle:

Serum alkaline phosphates hydrolyzes a colorless substrate of phenolphthalein monophosphate giving rise to phosphoric acid and phenolphthalein which, at alkaline pH values, turns into a pink color that can be determined through photometrics.

2 amino-2 methy1-1-propanol pH 11	7.9M
Phenolphthalein monophosphate	63M
Na_2HPO_4	80M
Stabilizers and preservatives	

Sample:
Serum or plasma with heparin
Procedure:

	Sample	Standard
Water	1.0	1.0
Substrate	1 drop	1 drop
Mix and incubate at 37˚C/20min		
Color developer	5.0	5.0
Reading:		
Blank	Water	
Color stability	a minimum of 1 hour	

***Calculations*:**

$$\frac{\text{SA O.D}}{\text{ST O.D}} \times 30 = \mu/1 \text{ of Alk. Phosphatase}$$

Normal values

Adults:9-35µ/1

Children: 35 – 100µ1

Determination for Bilirubin

Principle:

TOTAL BILIRUBIN (TB)
Pipette into Cuvette:

2.Sodium nitrate 25mmo1/1

Reagent 1	0.20	0.20
Reagent 2	-----	1 drop (0.05ml)
Reagent 3	1.00	1.00
Sample	0.20	0.20
Mix, and allow to stand for 10minutes at 20 -25˚C		
Reagent 4	1.00	1.00

Mix and allow a stand for 5 – 30minutes at 20 - 25˚C and then read the absorbance of the sample against the sample blank (ATB).

DIRECT BILIRUBIN

Pipette into Cuvette	Sample blank (m1)	Sample blank (m1)
Reagent 1	0.20	0.20
Reagent 2	-----	1 drop (0.05m1)
Sodium chloride (9g11)	2.00	2.00
Sample	0.20	0.20

Mix, and allow to stand for exactly 5 minutes at 20 -25˚C. read the absorbance of the sample against the sample blank (ADB).

Total bilirubin (mmo1/1)	=185 x A_{TB} (578nm)
Total bilirubin (mg/d1)	= 10.8x A_{TB} (578nm)
Direct bilirubin (mmol/1)	=246 x A_{DB} (546nm)
Direct bilirubin (mg/d1)	= 14.4 x A_{DB} (578nm)

Colorimetric method based on that described by jundrasisk and Grof (1938). Direct (conjugated) bilirubin reacts with Sulphanilic acid in alkaline medium to form a colored complex. Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized Sulphanilic acid.

Sample Collection and Preparation

Serum, plasma are the preferred samples. Fresh samples are usually kept out of direct light. Avoiding hemolysis as it interferes with the test.

1.Sulphanilic acid	29mmo1/1
Hydrochloric acid	0.17n
2.Sodium nitrate	25mmo1/1
3.Caffeine	0.26mo1/1
Sodium benzoate	0.52mo1/1
4.Tartrate	0.93mo1/1

Sodium hydroxide		1.9N
Procedure		
Wavelength:	Total bilirubin	578nm (560 – 600nm)
	Direct bilirubin	546nm (530 – 560nm)
	Cuvette	1cm light path
	Reaction temperature	20 -25˚C

Measurement: against sample blank

Statistical Analysis:

Result was presented as means ± standard error of mean for all values. Student 't' test was used for the test of significance between two values.

RESULTS

Table 3: Effect of Artesunate on serum Aspartate transaminase (AST), serum Alanine transaminase (ALT), serum Alkaline phosphatase (ALP) and Bilirubin in the Rats

GROUP AND DOSES OF ARTESUNATE	AST µmo1/1	ALT µmo1/1	ALP µmo1/1	BILIRUBIN mg/d1
Control (1) Normal water and feed.	51.40 ± 5.80	5.60 ± 0.87	652.05 ± 20.50	0.99 ± 0.080
Treated (2) (0.24mg/kg of artesunate)	58.60 ± 43.04	8.00 ± 1.13	592.02 ± 16 .50*	1.45 ± 0.125*
Treated (3) (0.34mg/kg of artesunate)	67.60 ± 21.43*	20.00 ± 2.49*	583.74 ± 32.47*	1.42 ± 0.156*
Treated (4) (76mg/kg of artesunate)	83.80 ± 18.14*	22.60 ± 3.64*	476.79 ± 1.15*	1.12 ± 0.143

Results are expressed as means ± standard error of mean (SEM) n = 5

* Significantly different from control at $p<0.05$

Table 2: Percentage weight difference of rats after four weeks treatment with artesunate

GROUPS	Mean Weight of Rats (g)		% Weight Difference
	BEFORE	AFTER	
Control (1)	130.18	184.70	41.88
Treated (2)	131.84	171.80	30.31
Treated (3)	133.20	180.54	35.54
Treated (4)	196.10	222.22	13.90

DISCUSSION

In Table 3, enzyme activities for both alkaline aminotransferase (ALT) and alkaline phosphatase (ALP) increased following doses of artesunate. The increase in enzymes activities were significant but not necessarily dose dependent at the dose level of 76mg/kg body weight of artesunate ($p<0.05$).For aspartate aminotransferase (AST), increases in enzyme activities at all dose levels of artesunate were apparent. The increases were significant at 0.34mg/kg and 76mg/kg body weight artesunate.

The dose dependent increases in serum enzyme activity of transaminases (ALT , AST) and the decrease in serum alkaline phosphatase (ALP) activity in addition to elevated levels of serum bilirubin suggests toxic effects on the liver leading to disruption or interference with routine liver functions[15]. The derangement appeared dose dependent. For ALP there were dose dependent decreases in enzyme activities suggesting that artesunate may have adversely affected ALP enzyme activity.

Dose dependent serum bilirubin level elevations was suggesting of impairment. This derangement though significant, may however not be consequent on extensive liver damage. The observed reduced enzyme activity at the highest dose levels, suggests an artesunate initiated inhibitory effect on the liver, which is likely dosage and exposure duration dependent.

Earlier studies have suggested that artesunate exerts adverse transient gut and neural related effects notably diarrhea, nausea or vomiting, stomach cramps or pain, loss of appetite, headache, itching, dizziness and sleep problems[16]. Embryolethality in rats has followed administration of artesunate [17]without affecting the maternal haematology[18].

Other studies point to adverse effects on the nervous system [19]and this reflect a wide range of symptoms that show different intensities of acute response to large doses or response to chronic ingestion of small to moderate doses of artesunate.

Table 4 shows dose-dependent decrease in percentage weight gain in rats. The results suggest that artesunate ingestion could lead to weight loss in the animals at all dose levels tested. Our results disagree with reports that artesunate was used to induce obesity in laboratory rats (Temidayo *et al* . , 2011).

CONCLUSION

High doses of artesunate may harm the liver as evidenced by increases in transaminase activities and increase in serum bilirubin levels. The adverse effects appeared to be dose dependent. It is therefore reasonable to suppose that normal doses of the drug which from our studies fall within the moderate range may not inflict severe injury on the liver.

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