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Acute administration of co-artesiane[®] induces oxidative stress in the testes of adult male Wistar rats

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(Received August 20, 2010; Accepted September 23, 2010)

ABSTRACT: Co-artesiane is an artemether-lumefantrine combination therapy that is used in the treatment of malaria. In this work we studied the toxicological effect of this drug with reference to seminal and biochemical parameters of the male reproductive system of wistar rats. Twenty (20) adult male rats were divided into four groups of five rats per group. Group A was administered a single dose (4 mg/kg/b.w) of Co-artesiane, Group B was administered double dose (8 mg/kg/b.w) of Co-artesiane and Group C received 10 mg/kg/ b.w of the drug for a period of 3 days. Group D served as control and received physiologic saline. In each group body weight, testicular weight, sperm count, motility and viability as well as oxidative stress status were assessed by evaluating the activities of reduced glutathione, Glutathione S-transferase, catalase, super oxide dismutase(SOD) and malondialdehyde (MDA). The mean sperm count, motility and viability in rats treated with Co-artesiane were reduced when compared with controls. Biochemical analyses showed increase in the activities of oxidative stress markers in a dose-dependent manner. The results suggest that the graded dose of Co-artesiane[®] elicit depletion of antioxidant defense system and induced oxidative stress in the rats.

Keywords: Anti-oxidant enzymes, Artemether-lumefantrine, Anti-fertility, Co-artesiane[®], Oxidative stress, Testes

Introduction

Malaria remains one of the most common causes of illness in the world, particularly in Africa and malaria chemotherapy has been linked with infertility. Artemether – Lumefantrine, an artemisinin –based combination therapy [ACT] is used as a therapeutic agent of choice in the treatment of malaria[1]. It is a new class of antimalarial indicated in the treatment of all forms of Plasmodium and multiple drug resistant strains of *P. falciparum*. Previous studies have shown that several classes of antimalarial drugs have been associated with adverse effects on reproductive functions in rodents. The aminoquinolines, chloroquine [2,3] quinoline – alcohols, quinine [4], Aryl–alcohol, halofantrine [5] and folate antagonist, sulphadoxine/pyrimethamine [6] have all been demonstrated to impact negatively on male fertility. Recent experimental studies on artemisinin derivatives such as artemether and dihydroartemisinin also reported significant decrease in sperm count, motility and testosterone level [7, 8].

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Besides, a number of studies have indicated that antimalarials alter antioxidant status. Rats exposed to quinine, amodiaquine, mefloquine and halofantrine exhibit an increased incidence of lipid peroxidation in the liver microsomal cells [9,10].

Chloroquine has been equally shown to impose oxidative stress on renal tissues [11] while halofantrine has been reported to cause increased lipid peroxidation and decreased enzyme and non-enzyme antioxidants [12, 13]. Various antimalarial drugs have been shown to exert different adverse effects; however, scanty information is available for artemether-induced potential side effects. Co-artesiane is an artemether-lumefantrine combination therapy commonly used in African countries in treatment of malaria caused by *Plasmodium falciparum*. This study was undertaken to examine the effects of Co-artesiane on testicular functions and its antioxidant defence system.

Materials and Methods

Animals

Twenty male wistar rats were purchased from the University of Ibadan. The weight ranged from 100g to 150g. They were randomised into four groups of five rats each and were housed in suspended wooden cages placed in a well-ventilated Animal House of the Department of Biochemistry, Bowen University, Iwo. They were fed with commercial rat pellets, had access to water *ad libitum*, and exposed to a natural daily photoperiodicity of 12 hours light and 12 hours darkness during the period of acclimatization and throughout the study.

Experimental Protocol

Animals were divided randomly into four groups of five rats each:

- Group A: Artemether / Lumefantrine combination (4 mg/24 mg/kg for 3 days);
- Group B: Artemether / Lumefantrine combination (8 mg/48 mg/kg b.w for 3 days);
- Group C: Artemether / Lumefantrine combination (10 mg/60 mg/kg b.w for 3 days)
- Group D: control (physiologic saline)

All procedures involving the use of animals in this study complied to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals [14].

Drug Administration

Co-Artesiane® (Dafra Pharma nv) powder consisting of 180 mg Artemether and 1080 mg Lumefantrine was purchased. The drug was prepared with a known mass of the Artemether - Lumefantrine powder suspended in distilled water to yield a suspension of 4 mg Artemether/24 mg Lumefantrine/ml, (8 mg Artemether/ 48 mg Lumefantrine /ml) and 10 mg Artemether/ 60 mg Lumefantrine/ml) respectively. The suspension was administered by oral gavage for a period of 3 days.

Body Weight

Body weights of the rats were measured before administration and at the end of the experiment by means of a weighing balance.

Oxidative Stress Markers

The levels of malondialdehyde (MDA), reduced glutathione, glutathione -S- transferase, catalase, superoxide dismutase, protein concentration were estimated in homogenates of the testes.

Seminal Analysis

At the end of the experiment, animals were sacrificed by decapitation and the caudal epididymides of the rats were incised and 5 μ L of epididymal fluid were delivered onto a glass slide, covered with a cover slip. Examination was done under light microscope at a magnification of $\times 400$. Sperm motility was also assessed immediately after sacrifice by conventional methods; motile spermatozoa were calculated per unit area and expressed as percentage motility. Epididymal sperm counts was made using the Neubauer improved haemocytometer and expressed as million/ml of suspension [15].

The sperm viability was also determined using Eosin/Nigrosin stain. Semen was smeared on microscopic slides and two drops of stain were added. Live (motile) sperm cells were stained while dead sperm cells remained unstained. Hundred sperm cells per slide were counted from a well prepared uniform smear in order to estimate the percentage live/dead ratio.

Homogenization

The testes were excised using scissors and forceps. They were trimmed of fatty tissue, washed in ice cold 1.15% potassium chloride solution, blotted with filter paper and weighed. They were then chopped into bits and homogenized in four volumes of the homogenizing buffer (pH 7.4) using a potter-elvehin homogenizer. The resulting homogenate was centrifuged at 10,000 g, 4 $^{\circ}$ C for 10 minutes to obtain the post-mitochondrial fraction. The supernatant was collected and then used for biochemical analysis.

Determination of Protein in the Samples

The post-mitochondrial fractions of the testicular supernatants were diluted 100 times with distilled water. This was done to reduce the sensitivity range of the Biuret method. 1 ml of the diluted sample was taken and added to 3 ml of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540 nm using distilled water as blank. The protein content of the samples were extrapolated from the standard curve and multiplied by 100 to get the actual amount in the fraction.

Assessment of Lipid Peroxidation

Lipid peroxidation was assessed by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation as described by Rice-Evans[16].

Glutathione Assay

The level of reduced glutathione (GSH) in the testes was determined by the method described by Buetler [17]. GSH concentration is proportional to the absorbance at 412 nm wavelength. All readings were taken within 5 minutes, as colour developed is not stable after that duration following the addition of Ellman's reagent.

Hydrogen Peroxide Assay

Hydrogen peroxide generation was determined according to the method of Wolff[18]. The assay mixture was thoroughly mixed by vortexing till it foamed. A pale pink colour complex is generated after incubating for 30 minutes at room temperature. The absorbance was read against blank (distilled water) at 560 nm wavelength.

Catalase Assay

Catalase activity was determined according to the method of Sinha[19]. After cooling at room temperature, the volume of the reaction mixture was made to 3 ml and the optical density measured with a spectrophotometer at 570 nm. The concentrations of the standard were plotted against absorbance.

Superoxide Dismutase Assay

The level of superoxide dismutase (SOD) activity was determined by the method of Mirsa and Fridovich[20]. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

Results

Table 1: Effect of Co-Artesiane on the body weight of male rats.

Treatment Groups	Initial weight	Final weight	% Difference
Group A	137.25 ± 2.12	138.40 ± 2.63*	0.83
Group B	141.93 ± 1.04	142.5 ± 1.98*	0.40
Group C	123.75 ± 2.23	124.10 ± 2.76*	0.28
Group D	130.10 ± 1.85	132.50 ± 1.64	1.85

Values are expressed as mean ± S.D, n=5

*Significantly lower compared to control at $P < 0.05$ when compared with control

Table 2: Effect of Co-Artesiane on Oxidative Stress Markers of the Testes in $\mu\text{mol/g}$ tissue.

Treatment Groups	Malonaldehyde	Reduced Glutathione	Glutathione-S- transferase	Catalase	Superoxide dismutase	Protein concentration
Group A	2.01±0.07	6.00±0.17	1.65±0.22 [†]	350.8±14.4 [#]	1.68±0.22 ⁺	2.08±0.10 [‡]
Group B	2.67±0.10*	5.95±0.07	1.52±0.21 [†]	301±20.7 [#]	1.44±0.11 ⁺	2.22±0.41 [‡]
Group C	2.09±0.16	5.85 ±0.07**	1.34 ±0.43 [†]	280.5 ±10.5 [#]	1.39 ±0.04 ⁺	2.65± 0.17 [‡]
Group D	1.97±0.09	6.07±0.02	2.26±0.14	453±21.2	1.88±0.13	1.58±0.28

Values are expressed as mean ± S.D, n=5

*Significantly higher than control at $P < 0.05$

** Significantly lower than control at $P < 0.05$

[†] Significantly lower than control at $P < 0.05$

[#] Significantly lower than control at $P < 0.05$

⁺ Significantly lower than control at $P < 0.05$

[‡] Significantly higher than control at $P < 0.05$

Group A: Artemether – Lumefantrine combination (4 mg/kg b.w for 3 days);
 Group B: Artemether – Lumefantrine combination (8 mg/kg b.w for 3 days);
 Group C: Artemether – Lumefantrine combination (10 mg/kg b.w for 3 days)
 Group D: Control (Physiologic saline);

Table 3: Effect of Co-Artesiane on testicular weight and seminal fluid parameters of the testes of wistar Rats.

Treatment Group	Testicular weight(g)	Sperm count ($\times 10^6$ /ml)	Sperm motility (%)	Sperm viability (%)
Group A	0.90 \pm 0.04	77.3 \pm 7.8*	62.5 \pm 9.6**	92 \pm 5.7
Group B	0.93 \pm 0.36	68.0 \pm 4.2*	60.0 \pm 8.2**	96 \pm 2.1
Group C	0.95 \pm 0.05	65.0 \pm 9.6*	62.5 \pm 9.6**	94 \pm 5.7
Group D	0.97 \pm 0.65	101 \pm 3.5	91.7 \pm 2.9	97 \pm 2.1

Values are expressed as mean \pm S.D, n=5

*Significant at $P < 0.05$ when compared with control

** Significant at $P < 0.05$ when compared with control

Body Weight

Table 1 above reveals that there was a general increase in body weight of the drug treated rats. Groups A, B and C showed increase of 0.83%, 0.40% and 0.20% respectively. However, weight gain was found to be statistically lower than control that showed an appreciation of 1.85%.

MDA

Table 2 shows percentage changes in the malondialdehyde (MDA) levels in the treated rats versus control. Testicular MDA levels were higher in all treatment groups but significantly so in Group C ($P < 0.05$).

Reduced Glutathione

Table 2 shows dose-dependent changes in the level of reduced glutathione in the treated rats versus control. Groups A, B and C had 1.2%, 2.0% and 3.8% decrease respectively when compare with control. The drop is significant in Group C.

Glutathione-S-transferase

Table 2 reveals dose dependent decrease in glutathione-S-transferase activity when compared with the control. The reduction is statistically different in all treatment groups when compared with the control.

Catalase

Table 2 reveals significant decrease in the activity of catalase in the treated rats versus control. Changes in catalase levels were comparative to the trend obtained for glutathione-S-transferase activity. After 3 days of treatment Groups A-C animals showed dose-dependent significant changes in catalase levels.

Superoxide dismutase (SOD)

Table 2 shows the activity of Superoxide dismutase in the testes of male rats treated with Co-artesiane. There was a significant, dose-dependent decrease in treated rats when compared with the control group.

Protein content

Table 2 reveals significant increase in the protein content in the Co-artesiane treated rats when compared with control (group of rats fed with pellets and water).

Testicular Weight and Seminar Parameters

Table 3 shows the effect of the drug on testicular weight and seminar fluid parameters. Results show that the sperm count and motility were significantly lower in the treated groups compared to control. Testicular weight and sperm viability were lower in the treated groups, though not significantly.

Discussion

We investigated the effects of acute administration of Co-artesiane on the oxidative stress status of the testes of adult male wistar rats. Results showed decrease in the levels of antioxidant enzymes reduced glutathione, Glutathione S-transferase, catalase, super oxide dismutase (SOD) and increase in the levels of MDA and protein content of the testes when compared with the control.

Artemether-lumefantrine has been reported to generate free radicals due to presence of an endoperoxide bridge [21] and anti-malarials in general have been reported to own their efficacy to their oxidant effect [22]. However free radicals are oxidants that have become a real concern because of their potential toxic effects, at high levels, on sperm function. Sikka [23]. A balance normally exists between free radicals [oxidants] production and antioxidant scavenging enzymes activities in the male reproductive system. As a result of such balance, only minimal amounts of oxidants remain, and they are needed for the regulation of normal sperm functions, such as sperm capacitation, the acrosome reaction, and sperm-oocyte fusion. The production of excessive amounts of oxidant in the testis can overwhelm the antioxidant defence mechanisms and causes oxidative stress, which decreases enzymatic defences of the testes and causes damage to spermatozoa thereby compromising sperm quality and functions [23,24].

In the present study lipid peroxidation indicated by high MDA level and Glutathione-S-transferase which are biomarkers of oxidative stress were increased in the rat given higher doses of Artemether - lumenfatrine for 3 days and glutathione level, superoxide dismutase and catalase were greatly reduced with higher doses (Table 2). The elevated level of malondialdehyde (MDA) in the testis of treated rat is a clear manifestation of excessive formation of free radicals and activation of lipid peroxidation systems. MDA is a good indication of the degree of lipid peroxidation and tissue damage. The MDA levels in treated rat significantly increased when compared with the control group. Superoxide dismutase is considered the first line of defense against deleterious effects of oxygen radicals in cell by catalyzing the dismutation of superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Superoxide dismutase protects catalase against inhibition by superoxide anion. Thus the balance of this enzyme system may be essential to get rid of superoxide anion and peroxides generated in subcellular compartments of the testis. The antioxidant enzymes, Superoxide dismutase and Catalase constitute a mutually supportive team of defence against reactive oxygen species. The dose-dependent reduction in SOD and catalase activities observed in this study is a clear indication of the oxidant capacity of the drug and supports previous reports that chloroquine, an anti-malaria, inhibited catalase and peroxidase activities [25].

There was an insignificant decrease in the testicular weight of treated rats. This reflects the extent of degeneration caused by the drug and might have resulted from reduced tubule size. It might also reflect a combination of the toxicity of artemether-lumefantrine on testicular function.

The marked azoospermia and reduced motility and viability in treated rats might be due to spermatogenic arrest and inhibition of steroid biosynthesis of leydig cells induced by reactive oxygen species (ROS). This is because mammalian spermatozoa are rich in polyunsaturated fatty acids and, thus, are very vulnerable to ROS attack which results in decreased sperm motility, decreased sperm viability, and increased midpiece morphology defects with deleterious effects on sperm capacitation and acrosome reaction [26]. Lipid peroxidation of sperm membrane is considered to be the key mechanism of this ROS-induced sperm damage leading to infertility [27].

Conclusion

We conclude that Co-artesiane, an artemether-lumefantrine combination depleted antioxidant system and increased lipid peroxidation by reactive oxygen species generated by the compound, thereby causing oxidative stress in the testes of rats; and that this is dependent on the dosage, duration and its ability to infiltrate the blood-testis barrier. The results obtained in this work are consistent with the reported anti-fertility effects of anti-malarials.

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