Journal of Morphological Sciences
J Morphol Sci
Brazilian Society of Anatomy

Original Article
J Morphol Sci 2023;40:331-342

Oral Artemisinin-Based Combination Therapies Induced Gonadotoxicities and Diminished Prostate Specific Antigen (PSA) in Wistar Rats

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Disclose and conflicts of interest: none to be declared by all authors

ABSTRACT

Introduction: testicular histomorphology and hormonal alterations have been reported in few artemisinin-based combination therapies (ACTs) used for treatment of malaria. We are first to comparatively characterize possible gonadotoxicities from seven commercially available ACTs in an experimental model. Forty adult male Wistar rats were allotted (n = 8) groups of animals (n = 5) each. Group 1, normal control (NC) received placebo, while Groups 2 to 8 received oral therapeutic combined doses of artesunate-amodiaquine (AA) 5.71 mg, artesunate-mefloquine (AM) 19.29 mg, artesunate-sulfadoxine-pyrimethamine (ASP) 10.35 mg, artesunate-pyronaridine (APy) 10.29 mg, artemisinin-piperaquine (AP) 12.5 mg, dihydroartemisinin-piperaquine (DP) 15.42 mg, and artemether-lumefantrine (AL) 8 mg respectively for three days, except AP administered for two days (standard regimen). Animals were euthanized 24 hours after last administration under ketamine-xylazine anesthesia. Serum and testes were analyzed for hormonal and histomorphological alterations. Testosterone demonstrated increasing concentration with significant ($p < 0.05$) increase in AA, APy and AP-administered groups with corresponding decrease in prostate specific antigen. Sperm counts and motility significantly ($p < 0.05$) decreased in ACT-administered groups. Antioxidants catalase and superoxide-dismutase levels in ASP, APy and DP-administered groups significantly decreased in correlation with elevated oxidative stress markers in ASP, APy and DP-administered groups. Widening of germinal epithelium, basement membrane distortion and spermatogonial cells degeneration were prominently demonstrated in ASP, APy, AP and DP-administered groups. In conclusion, oral therapeutic exposures to ACTs elicited gonadotoxicity with testiculo-microstructural, hormonal and antioxidant alterations ranging from mild to severe; AA, AP, AL, AM, APy, DP to ASP, respectively and generally decreasing PSA level.

Keywords: Artemisinin-based combination therapy; Hormones; Oxidative stress; Seminal analysis; Testis; Histology.

Introduction

Malaria is the most prevalent parasite disease that globally affects about 3.3 billion people in 106 countries[1], with the African region accounting for approximately 90% of malaria-associated morbidity and the second leading cause of deaths in Africa after HIV/AIDS[2,3]. Malaria is an acute infectious disease caused by five species of the protozoal genus *Plasmodium* (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*)[4,5]. The greatest prevalence of malaria morbidity and mortality recorded worldwide is associated with *P. falciparum* infection[2,6]. It is transmitted to humans through the bite of a female anopheles' mosquito, which thrives mostly in humid and swampy areas[7]. *P. falciparum* is responsible for causing an acute, rapidly fulminating disease that is characterized by persistent high fever, orthostatic hypotension, and massive erythrocytosis[2,3]. Malaria-associated multiple organ dysfunctions are widely reported, and with deleterious effects on the male gonad and its severity may induce liver dysfunction[8], acute renal failure[9] and cerebral malaria[10].

Artemisinin-based combination therapies (ACTs) are the recommended first line medication for the treatment of uncomplicated malaria, while intravenous quinine or artesunate (monotherapy) is often used in the mitigation of complicated malaria, in both endemic and non-endemic countries[2,11]. These ACTs include; artesunate-amodiaquine, artesunate-mefloquine, artesunate with sulfadoxine-pyrimethamine, artesunate-pyronaridine, artemisinin-piperaquine, dihydroartemisinin-piperaquine, and artemether-lumefantrine, respectively. Altogether, they serve as

DOI: 10.51929/jms.40.331.2023
ISSN 2177-0298

the main chemotherapeutic agents against malaria parasites[2,3], and possess greater efficacy in treating malaria infection, reducing transmission in endemic areas and producing lower levels of re-infection[12,13].

However, prolong use of ACTs have been associated with adverse effect in the male reproductive functions which include; anti-fertility activity, increase in abnormal sperm cell, sperm counts and motility reduction[14-16]. Artesunate has been reported to cause a degenerative effect on rat testes[17], and alter testicular weights[17,18]. Artesunate-amodiaquine and artesimisinin-piperaquine caused a marked increase in testosterone levels compared to the control groups[19]. Therapeutic combinations of artesunate-amodiaquine and artesunate-sulfadoxine-pyrimethamine reportedly distorted sperm morphology, and reduced sperm count and motility[19,20]. Artesunate at 4 mg/kg b.w and 8mg/kg b.w may cause inflammation and aggravate male infertility[21]. However, Edagha *et al*.,[22] and Mukherjee *et al*.,[23] had reported that oral therapeutic exposures to some ACTs ameliorated severe *Plasmodium berghei*-induced gonadotoxicity in Swiss mice.

Likewise, it has been reported that African-American men have the highest incidence of prostate cancer (Pca) worldwide and more prone to develop the disease earlier in life than any other racial and ethnic groups[24,25]. African-American men have incidence rates of Pca that is about 40 times higher than those in Africa[24,26]. These differences may not be unconnected with the degree of dependence of these continental populations on herbal medicine[27]. Environmental factors reportedly play an important role in the etiology of the Pca, and the variations in their incidence. Thus correlating with report that the incidence rates of Pca are lower in Africa and Asia than those of developed countries[28] which some have ascribed to rate of PSA testing[28,29].

Artemisia annua L. is a Chinese medicinal plant, widely consumed as tea or press juice to treat malaria throughout Asia and Africa. Artemisinin and its derivatives (dihydroartemisinin, artesunate, artemether, arteether) have been found to exert profound activity against tumors including Pca cells[30,31]. No previous study has comparatively characterized the gonadotoxic effect of all available ACTs recommended for malaria treatment. We hypothesized that the standard regimen of oral therapeutic exposure to the available seven ACTs in Nigeria, induce gonadotoxicity, but may alter serum PSA concentrations in adult male Wistar rats.

Materials and Methods

Ethical Approval

This study was approved by the Department of Human Anatomy Ethics Committee in accordance with the Principle of Laboratory Animal Care and Use[32] and granted approval by the Health Research Ethics Committee of the Akwa Ibom State Ministry of Health with Ref: MH/PRS/99/Vol.IV/696.

Experimental Design

All study animals were allotted into eight groups of five rats each wherein Group 1 was the normal control (NC), and received placebo (5 mL distilled water per kg body weight), whereas Groups 2 to 8 received therapeutic combined doses of artesunate-amodiaquine (AA) 5.71 mg; artesunate-mefloquine (AM) 19.29 mg; artesunate-sulfadoxine-pyrimethamine (ASP) 10.35 mg; artesunate-pyronaridine (APy) 10.29 mg; artemisinin-piperaquine (AP) 12.5 mg; dihydroartemisinin-piperaquine (DP) 15.42 mg; and artemether-lumefantrine (AL) 8 mg per kg body weights respectively for three days via oral route except AP which was administered for two days (standard regimen). The experiment was conducted between 8:00 am and 10:00 am, and 4:00 pm to 6:00 pm for groups that involved repeat doses.

Experimental Animals

Forty (40) matured male Wistar rats weighing between 180 - 220 g were obtained from the Animal House, Department of Zoology, Faculty of Science, University of Uyo, Nigeria. The animals were housed in standard cages with dimensions 20 cm long, 20 cm wide and 15 cm high. Five rats were kept in a cage, under controlled environmental conditions (25 °C and a 12-h light/dark cycle) and had free access to standard rat pellet food (Vital® Feed) and tap water throughout the experiment. They were allowed to acclimatize for two weeks prior to the commencement of the study.

Drug Acquisition and Preparation

The ACTs (seven popular brands in Nigeria) were used in this study, namely; Camosunate® (artesunate-amodiaquine lot number: 120917) manufacturer Geeith Pharmaceutical Limited; Artequin® (artesunate-mefloquine lot number: 1750804) manufacturer Ateco Switzerland; CoArinate® (artesunate-sulfadoxine-pyrimethamine) batch number: 279) manufacturer Famar, Italy; Artequick® (artemisinin-piperaquine lot number: 20180703) manufacturer Artepharm Co. Ltd, China; P-Alaxin® (dihydroartemisinin-piperaquine: lot number H1AFN- 102) manufacturer Blissars Pharm Ltd, India; Pyramex® (artesunate-pyronaridine lot number: U003) manufacturer Shin Poong Pharmaceutical Co. Ltd, Korea; and Coartem® (artemether-lumefantrine lot number: KF167) manufacturer Novartis Pharmaceutical Limited, Switzerland. All drugs were checked for expiration dates, and verified as authentic.

The solid drug tablets were pulverized in a ceramic mortar and pestle until smooth powder, and diluted in 400 mL of water measured out with a measuring cylinder and administered appropriate volume orally with an oro-gavage cannula.

Drug Administration

The dosage of the drugs administered was determined by calculating:

Weight of animal/1000 × Dosage/stock.

Each drug dosage was based on the weight of a physiologic man, at 70 kg. The stock was equally derived by taking each drug dosage to the quantity of water used to dilute the drug which was 400 mL. Appropriate tubing size for oral administration were strictly adhered to just minimize administration discomfort[33].

The rats were weighed on the first day of the experiment and marked with markers to specify each weight for dosage and to avoid mistakes during administration. The final weights were taken on the last day after administration in the morning between 7:00 am and 9:00 am using electric balance (Zeiss, West Germany (Pty) Ltd; 0.000g).

Animal Sacrifice and Collection of Samples

The experimental animals were sacrificed under ketamine (40 mg/kg)/xylazine (5 mg/kg) anesthesia (Sigma Aldrich, Germany), 24 hrs after last drug administration. Blood was collected via intracardiac puncture, and 2 mL of blood obtained in a plain tube and centrifuged at 4000 rpm for 15 minutes using an MSE top centrifuge® to obtain serum for biochemical assay. The testes were harvested and preserved in 10 % Bouin's fluid for 6 hours, then transferred to 70 % alcohol prior to histological evaluation[34]. The testes were excised and separated from their cauda epididymis. Each testis was weighed using an electronic balance (Mehler Toledo; Microstep (Pty) Ltd; Circifense, Switzerland), the average of the two testes for each animal was recorded.

Determination of Testicular Morphometry

Each testis was weighed using an electronic balance and the volumes were taken with a measuring cylinder. The testicular diameters and lengths were obtained with an electronic Vernier caliper and (Mehler Toledo; Microstep [Pty] Ltd; Circifense, Switzerland) the average of the two testes for each animal was recorded.

Determination of Reproductive Hormones

The serum concentrations of luteinizing hormone ([LH] EIA - 6K4J9), testosterone (EIA - 37K219), follicle stimulating hormone ([FSH] EIA - 4K1I9) and prostate specific antigen ([PSA] EIA - 37K219) were determined using Elabscience Enzyme Immunoassay (ELISA) rats specific kits with catalog number CA92630 USA purchased from CE Partners 4UBV Esdoorniaan 133951DB Maain, The Netherlands. Monobind Inc. 100 North Painte Dr. Lake Forest.

Determination of Testosterone Concentration

This was done by securing desired number of wells with the samples. Ten microliter (10 mL) of standards, specimens and control were dispensed into appropriate wells. Working testosterone enzyme reagents (50 µL) was dispensed into each well, swirled thoroughly and allowed to mix for 20 - 30 seconds. Testosterone biotin reagent (50 µL) was also dispensed into each well, swirled thoroughly and allowed to mix for 20 - 30 seconds. The mixture was allowed to incubate for 60 minutes at room temperature. The contents of the microwells were discarded by decantation, then rinsed and flicked 3 times with wash buffer (350 µL). Working substrate solution (100 µL) was dispensed to each well. The mixture was incubated for 15 minutes, and the reaction was stopped by addition of stop solution (50 µL) to each well and gently mixed for 15 - 20 seconds to ensure a complete colour change from a brown to yellow solution. Absorbance at 450 nm was read within 30 minutes with a microplate reader[35].

Determination of Follicle Stimulating Hormone Concentration

The desired number of coated wells was secured in a holder. Fifty micoliter (50 µL) of standards specimens and control were dispensed into appropriate wells. The FSH enzyme reagent solution (100 µL) was dispensed into each well, swirled thoroughly and allowed to mix for 20 - 30 seconds. The mixture was allowed to incubate for 60 minutes at room temperature. The content of the microwells were discarded by decantation then rinsed and flicked three times with wash buffer (350 µL). Working substrate solution (100 µL) was dispensed to each well. The mixture was incubated at room temperature for 15 minutes. The reaction was stopped by addition of stop solution (50 µL) to each well and gently mixed for 15 - 20 seconds to ensure a complete colour change. Absorbance at 450 nm was read within 30 minutes with a microplate reader[36,37].

Determination of Luteinizing Hormone Concentration

Fifty microliter (50 µL) of standards, specimens and control were dispensed into appropriate wells. The LH enzyme reagent (100 µL) was dispensed into each well swirled thoroughly and allowed to incubate for 60 minutes at room temperature. The contents of the microwells were discarded by decantation, then rinsed and flicked three times with wash buffer (350 µL). Working substrate solution (110 µL) was dispensed to each well and gently mixed for 15 - 20 seconds to ensure a complete colour change. Absorbance at 450 nm was read within 30 minutes with microplate reader[37].

Determination of Total Prostate Specific Antigen (tPSA) Concentration

This was done by securing the number of wells, 50 µL of standards; specimens and control were dispensed into appropriate wells. The tPSA enzyme reagent (100 µL) was dispensed into each well, swirled thoroughly and allowed to mix for 20 - 30 seconds.

The mixture was allowed to incubate for 60 minutes at room temperature. The contents of the microwells were discarded by decantation, then rinsed and flicked 3 times with wash buffer (350 µL). Working substrate solution (100 µL) was dispensed to each well. The mixture was incubated at room temperature for 15 minutes. The reaction was stopped by addition of stop solution (50 µL) to each well and gently mixed for 15 - 20 seconds to ensure a complete colour change. Absorbance at 450 was read within 30 minutes with a microplate reader.

Seminal Analysis

Normal saline 5 mL was utilized to mince the harvested cauda epididymis from each animal and thereafter used for sperm count, motility and morphology determination respectively. 50 µL of the prepared epididymal spermatozoa was diluted in 950 µL diluents mixed in pipette and put on a Hemocytometer and covered with a cover slip (22 × 22 mm) for immediate viewing under the light microscope objective adjusted to x40 magnification. Each field was scanned systematically and repeatedly for determination of sperm counts, motility and morphology of the spermatozoa. Counting was repeated in each chamber and average count documented. Average number of cells, their density, dilution factor, and cell concentration was then calculated for by applying the formula as described for a similar reproductive toxicity as described by Osinubi *et al.*[38], and Cooper *et al.*[39],. Staining dry smeared diluted epididymal fluid on a glass slide with eosin-nigrosine, the sperm morphology was accessed under a light microscope (Leica Dm 500) at x40 magnification. The number of normal spermatozoa, as well as spermatozoa with abnormal heads, tails, and mid-piece were recorded in percentage[40].

Oxidative Stress in Testes

Superoxide dismutase and catalase were the antioxidants assayed in this study, together with reduced glutathione and malondialdehyde (indices of lipid peroxidation). The animals were dissected to access the testes. In order to derive the post mitochondrial fraction, testes were washed in ice cold 1.15% KCl solution, blotted, weighed and then homogenized in 0.1 M phosphate buffer (pH 7.2). The homogenate derived was centrifuged for 15 min (at 2500 rpm) and the supernatant decanted and stored at - 20 °C for analyses. The malondialdehyde (MDA) levels (being an index for lipid peroxidation) were estimated as described by Chen and Zhang[41]. Reduced glutathione (GSH) concentration (a non-protein sulphydryl) in testicular tissue was determined as described by Ellman reagent/DTNB[42,43]. Catalase activity was estimated by the method of Singha[44], while superoxide dismutase (SOD) was determined as described by Mishra and Fridovich[45] using its ability to inhibit auto-oxidation of epinephrine (whose absorbance increases at 480 nm).

Histopathological Assessments

The harvested testes were initially fixed in Bouin's fluid for six hours before transferring into 70 % alcohol for 24 hours[34]. The tissue samples were thereafter passed through graded ethanol series, cleared, infiltrated in paraffin wax and sectioned at 5 µm with a Leica RM2125 RTS microtome. The albumenized tissue slides were stained with haematoxylin and counterstained with eosin[46]. The photomicrographs were thereafter obtained from evaluation of at least five fields/sections using light microscope (Olympus-CX31) attached to AmScope® digital camera (MU 1000) China, and interpreted by at least three independent histopathologists to mitigate against bias.

Statistical Analysis

The software, IBM for Statistical Package for Social Science (SPSS) version 25 and Microsoft Excel were utilized to perform the statistical analyses. A comparison among groups was performed using a One-Way Analysis of Variance (ANOVA), and the results subjected to post-hoc analysis with Turkey multiple-comparison test. All data were expressed as mean ± standard error, and the values of $p < 0.05$ were considered significant.

Results

The Comparative Effect of Oral Administration of ACTs on Testiculo-morphometry

The body weight showed an increasing trend throughout the test groups compared to NC, except in the NC and AA-administered groups. The AP and AL groups showed markedly increased weight gain compared to NC (Table 1).

Testicular weights showed a significant ($p < 0.05$) increase in APy-administered group compared to NC, AA, ASP and AL-administered groups. The testicular volume showed a significant increase in AA, AM and APy compared to NC (Table 1).

The comparative effect of oral administration of ACTs on testicular hormones

Serum testosterone concentrations showed a non-significant increase in all the ACT-administered groups except AA when compared with NC (Table 2). The FSH concentrations were significantly decreased in AA, ASP, DP and AL-administered groups compared to NC group, whereas the LH concentration was deccrease in administered group except AL-administered group. Conversely, there was slight decrease in the concentration of PSA across the ACT-administered groups when compared to NC (Table 2).

The Comparative Effect of Oral Administration of ACTs on Oxidative Stress

The brain enzymatic antioxidant; superoxide dismutase(SOD) was signicantly decreased in all ACT-administered groups compared to NC, and specifically ASP, APy and DP-administered groups had significantly decreased SOD compared to AA, AP, AM

and AL-administered groups (Fig. 1). Catalase was significantly decreased in ASP-administered group compared to all other test groups and the normal control (Fig. 2).

The reduced glutathione (GSH) concentration was significantly increased in the ASP-administered group compared to NC, AA, AM, AP and AL-administered groups (Fig. 3). Likewise the malondialdehyde (MDA) concentrations increase in all ACT-administered groups and with significant increased in the ASP, APy and DP-administered groups compared to NC (Fig. 4).

The Comparative Effect of Oral Administration of Acts on Sperm Count and Motility

The sperm counts were significantly ($p < 0.05$) decreased in all the ACT-administered groups compared to normal control (NC) group. Likewise, there was significant decrease within the ACT-administered groups compared to AA group (Fig. 5).

There was marked percentage decrease in the number of active spermatozoa (motility) across all the ACT-administered groups, except in AA compared with NC, while the inactive and sluggish spermatozoa increased the across ACT-administered groups compared to NC (Fig. 6).

The Comparative Effect of Oral Administration of Acts on Testicular Histology

Sections through the seminiferous tubules of the Normal control groups (NC) demonstrated good circular morphology of normal appearing seminiferous epithelium with the presence of well organized spermatogenic lineage series and spermatozoa within in the lumen (Fig. 7). The ACT-administered groups generally demonstrated degrees of reduction of cells within the spermatogogenic series, widening of the tubular lumen, tubular atrophy, prominent, distortion of interstitium, hypocellularity and decreased spermatozoa in tubular lumen. The ASP, APy and DP-administered groups had severe distortions; these alterations were moderate in the AM and AP-administered groups while there were mild degenerations observed in AA and AL-administered groups (Fig. 7).

Table 1. Comparative effect of oral administration of ACTs on testiculo-morphometry of Wistar rats.

Experimental Group	Initial BW (g)	Final BW (g)	Weight gain (g)	Testicular Weight (g)		Testicular Volume (mL)	
				Right	Left	Right	Left
NC	130.20 ± 3.84	132.80 ± 6.04	2.60 ± 4.57	1.07 ± 0.04	1.02 ± 0.03	0.36 ± 0.02	0.43 ± 0.02
AA	168.60 ± 10.75	172.20 ± 12.01	3.60 ± 2.06	1.23 ± 0.01	1.23 ± 0.01	0.68 ± 0.02[a]	0.62 ± 0.02
AM	218.00 ± 25.77	224.40 ± 28.71	6.40 ± 3.99	1.51± 0.12	1.48 ± 0.11	0.68 ± 0.08[a]	0.68 ± 0.08[b]
ASP	155.60 ± 7.16	165.40 ± 6.59	9.80 ± 1.36	1.06 ± 0.09	1.07 ± 0.09	0.42 ± 0.05	0.40 ± 0.06
APy	193. 60 ± 14.40	200.40 ± 15.66	6.80 ± 3.17	1.61 ± 0.05***	1.68 ± 0.09***	0.68 ± 0.07[a]	0.68 ± 0.04[b]
AP	238.00 ± 13.36	254.40 ± 10.97	15.60 ± 2.94	1.48 ± 0.05	1.49 ± 0.05	0.60 ± 0.03	0.56 ± 0.04
DP	158.40 ± 7.67	166.80 ± 6.48	8.40 ± 3.50	1.55 ± 0.07	1.42 ± 0.09	0.58 ± 0.04	0.52 ± 0.04
AL	161.80 ± 5.09	173.20 ± 6.37	11.40 ± 1.72	1.42 ± 0.05	1.39 ± 0.04	0.58 ± 0.02	0.58 ± 0.04
P value	-	-	-	0.0001	0.0001	0.0001	0.0001

Values are Mean ± Standard Error (n = 5 animals per group). $p < 0.05$ indicate statistically significant values between the groups. *** = significantly increased compared NC, AA, ASP and AL; a = significantly increased compared NC, b = significantly increased compared NC and ASP. NC = normal control, AA = artesunate-amodiaquine, AM = artesunate-mefloquine, ASP = artesunate-sulfadoxine-pyrimethamine, APy = artesunate-pyronaridine, AP = artemisinin-piperaquine, DP = dihydroartemisinin-iperaquine, AL = artemether-lumefantrine. BW = body weight, and TW = testicular weight.

Table 2. Comparative effect of oral administration of ACTs on testicular hormones of Wistar rats.

Experimental Group	Testicular Hormones			
	Testosterone (mg/mL)	FSH (mIU/mL)	LH (mIU/mL)	PSA (mg/mL)
NC	1.49 ± 0.14	2.15 ± 0.22	1.24 ± 0.16	0.64 ± 0.07
AA	1.48 ± 0.16	1.21 ± 0.14*	0.79 ± 0.11	0.39 ± 0.07
AM	1.87 ± 0.21	1.43 ± 0.10	0.77 ± 0.09[a]	0.38 ± 0.01
ASP	1.50 ± 0.13	1.05 ± 0.17*	0.89 ± 0.10	0.41 ± 0.04
APy	1.65 ± 0.30	1.46 ± 0.11	1.03 ± 0.08	0.47 ± 0.03
AP	2.12 ± 0.18	1.52 ± 0.32	0.79 ± 0.09	0.57 ± 0.14
DP	1.83 ± 0.31	1.16 ± 0.11*	1.16 ± 0.11	0.36 ± 0.03[b]
AL	1.65 ± 0.23	1.27 ± 0.22*	0.80 ± 0.23	0.38 ± 0.04
P-value	0.392	0.011	0.027	0.048

Values are expressed as Mean ± Standard Error (n = 5). * = $p < 0.05$ relative to NC; a = $p < 0.05$ relative to NC; b = $p < 0.05$ relative to NC (group 1).
Legend: AA = artesunate-amodiaquine 5.71 mg/kg body weight (bw); AM = artesunate-mefloquine 19.29 mg/kg bw; ASP = artesunate-sulfadoxine-pyrimethamine 10.35 mg/kg bw; APy = artesunate-pyronaridine 10.29 mg/kg bw; AP = artemisinin-piperaquine 12.5 mg/kg bw; DP = dihydroartemisinin-piperaquine 15.42 mg/kg bw; and AL = artemether-lumefantrine (AL) 8 mg/kg bw.

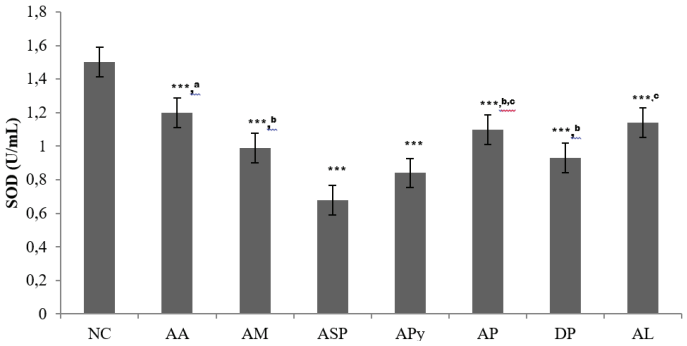

Figure 1. Comparative effect of oral administration of ACTs on superoxide dismutase in Wistar rats.
Legend:
*** = AA, AM, ASP, APy, AP, DP and AL significantly decreased compared to NC;
[a] = ASP, APy and DP significantly decreased compared to AA;
[b] = AM, DP and AP significantly increased compared ASP;
[c] = AP and AL significantly increased compared to APy.

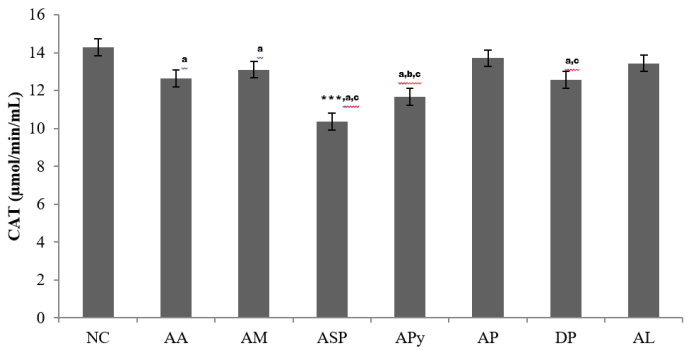

Figure 2. Comparative effect of oral administration of ACTs on catalase (CAT) in Wistar rats.
Legend:
*** = NC, AA, AM, APy, AP, DP and AL significantly increased compared to ASP;
[a] = AA, AM, ASP, APy and DP significantly decreased compared to NC;
[b] = APy significantly decreased compared to AM;
[c] = ASP, APy and DP significantly decreased compared to AP.

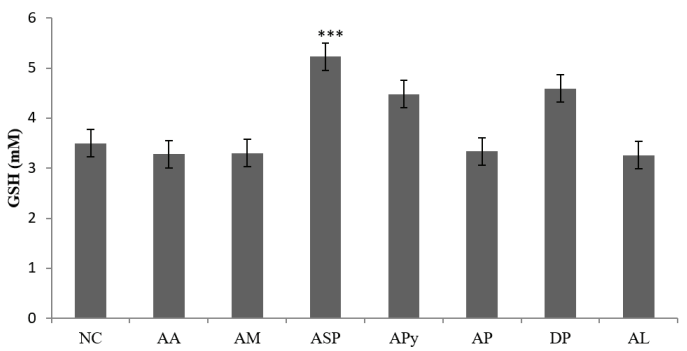

Figure 3. Comparative effect of oral administration of ACTs on the reduced glutathione (GSH) of Wistar rats.
Legend:
*** = ASP significantly increased compared to NC, AA, AM, AP and AL.

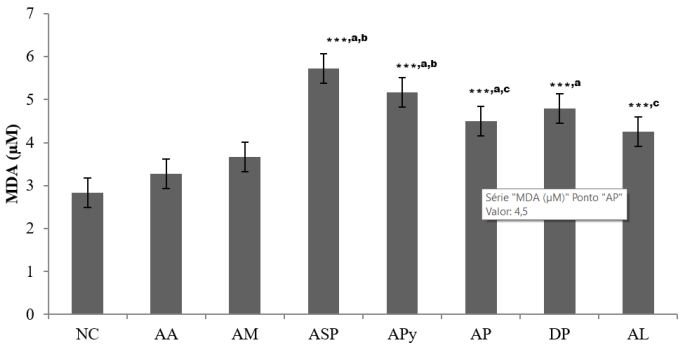

Figure 4. Comparative effect of oral administration of ACTs on malondialdehyde (MDA) in Wistar rats.
Legend:
*** = ASP, APy, AP, DP and AL significantly increased compared to NC;
[a] = ASP, APy, AP and DP significantly increased compared to AA;
[b] = ASP, APy and DP significantly increased compared to AM;
[c] = AP and AL significantly decreased compared to ASP.

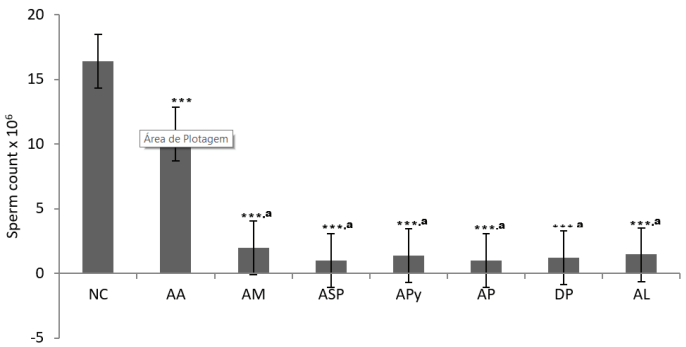

Figure 5. Comparative effect of oral administration of ACTs on sperm counts of Wistar rats
Legend:
*** = AA, AM, ASP, APy, AP, DP and AL significantly decreased compared to NC;
[a] = AM, ASP, APy, AP, DP and AL significantly decreased compared AA.

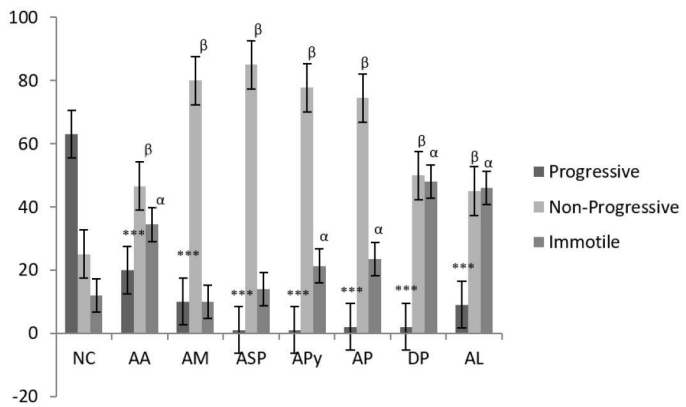

Figure 6. Comparative effect of oral administration of ACTs on sperm motility of Wistar rats
Legend:
*** = AA, AM, ASP, APy, AP, DP and AL significantly decreased compared to NC;
β = AA, AM, ASP, APy, AP, DP and AL significantly increased compared NC;
α= AA, APy, AP, DP and AL significantly increased compared to NC.

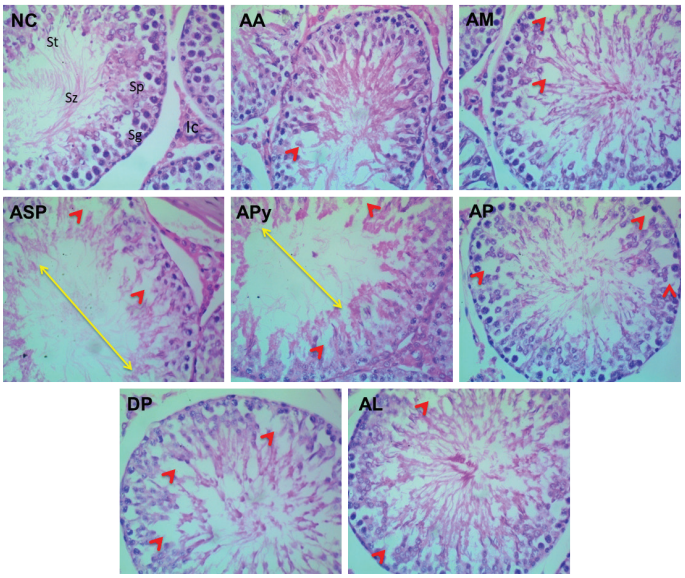

Figure 7. Representative photomicrographs of the cross section of the testes of normal control (NC) the group 1, and test groups 2 to 8 ([artesunate+amodiaquine = AA], [artesunate+mefloquine = AM], [artesunate+pyronaridine = APy]; [artesunate-sulfadoxine+pyrimethamine = ASP], [artemisinin+Piperaquine = AP]; [dihydroartemisinin+piperaquine = DP]; and [artemether+lumefantrine] respectively) H&E x400. Yellow arrows = widening of tubular lumen; Red arrow head = atrophy and degenerated spermatogenic series.

Discussion

Malaria is a major public health problem especially in African countries affecting over 500 million people annually, and the ACTs are used in both endemic and non-endemic countries as first line medication for the treatment of uncomplicated malaria[2,11,47]. Report has shown that ACTs possess greater efficacy in treating malaria infection, reducing transmission and producing lower levels of re-infection[12,13,22], and also several report has shown that ACTs has negative effect on different cells and organs of the body including the testes and prostrate[14,16-19].

Malaria parasite act by digesting hemoglobin within its acidic food vacuole, leading to the release of toxic ferriprotoporphyrin IX (FP) and reactive oxygen species[46,47]. Ferriprotoporphyrin IX interract with phospholipid membranes to cause structural effect due to its Fe^{3+}reactivity with unsaturated membrane lipids, leading to increased membrane permeability for ions, cell swelling, and lysis[48-52].

In ACTs therapy, artemisinin, the parent drug, has a more potent dihydroartemisinin and its derivatives, artemether, artemotil, and artesunate respectively. There have an oral absorption bioavailability (> 60%) and with peak concentrations usually achieved within 4 hours[53,54]. Although the bioavailability and safety of the ACTs is dependent on their partner drugs[53-56].

Artemisinin and its derivatives are the most rapidly eliminated of all anti-malarials with half-lives of approximately 1 hour. The slow elimination of the partner drug allows 3-day regimens to be given[57-59]. Due to the rapid elimination of artemisinin derivatives, there is complete protection for these derivatives and most toxicities are associated with the long half life of the partner drugs[59]. It has been reported the mechanism of action of artemisinin involves the cleavage of its endoperoxide bridge forming C-centered radicals through free heme which react will cellular targets such as enzymes and membrane lipids[60,61]. Also, artemisinin-heme adducts inhibit heme polymerization in a mechanism that involves parasite's histidine-rich proteins-II and III and that this inhibition most likely leads to free accumulation of the toxic ferriprotoporphyrin IX (FP) and eventually form artemisinin-FP adducts and ROS that induces membrane damage and eventually parasite death[60-63].

Artemisinin is also involved in the inhibition of parasite mitochondria (complex-IV cytochrome-C oxidase) through production of reactive oxygen species, with complex-IV activating artemisinins through transfer of electrons to the endoperoxide[48,63,64]. Generation of oxidative stress in antimalaria therapy is important in malaria parasite clearance as anti-malarial drugs, and ACTs are thought to act by increasing cellular oxidative stress[48,63].

The blood testes barrier (BTB) consist of mostly tight junctions (TJs) between endothelial cells, and in humans is constituted of *coexisting* specialized junctions between Sertoli cells near the basement membrane, which include TJs, basal ectoplasmic specializations (basal ESs), gap junctions (GJs), and desmosome-like junctions[65,66]. This barrier, segregate the seminiferous epithelium anatomically into apical and basal compartment and helps determines what nutrients, electrolytes, ions and others can penetrate into the testes[65,66]. Different substance penetrate the blood testes barrier via transporters and about 60% of drug transporters known to exist are made up of integral membrane proteins that transport or prevent the transport of xenobiotics across biological membranes in cell epithelia and endothelia in mammals [66-68]. Drug transporters can be classified as either as primary active transporters that require ATP hydrolysis for function (ATP-binding cassette (ABC)), or as secondary and tertiary active transporters driven by ion exchange or co-transport without the consumption of ATP (solute carrier (SLC)) [66,69]. ACTs utilizes ABC transporters to penetrate the blood testes barrier using either multidrug resistance proteins (MDRs), MDR-related proteins (MRPs), and breast cancer resistance protein (BCRP)[66,69-72].

The MDA level indicates the degree of lipid peroxidation and tissue damage[72-74]. The MDA levels in the ACT-administered groups significantly increased when compared with the control group as shown in Fig. 4. This finding agrees with the report of Akoriku and mankwe[14], and Onyeneka *et al*[73]. It is suggested and evident that artemisinin generates free radicals to kill malaria parasites[73,74]. Also, sodium artesunate a metabolite of artemisinin derivatives has been reported to increase the level of active oxygen species and

production of malondialdehyde in normal red blood cells and to a greater extent in malaria infected red blood cells[73,74] as shown in ASP, APy and DP- administered groups when compared to control group and AP, DP, AM and AL test. This may be due to the increase bioavaialability of artesunate and dihydroartemisinin and their ability to penetrate the BTB[66,75]. This study agree with the findings of Chiagoziem *et al.*[76] that artemether-lumefantrine-(1.14/6.86 mg/kg/d) and artesunate-amodiaquine-(2.86/8.58 mg/kg/d) increase MDA levelindicating a clear manifestation of excessive formation of free radicals and activation of lipid peroxidation systems[5,77].

The decrease in levels of SOD and catalase in ACT-administered groups was significant in ASP, APy and DP-administered groups compared to control group as shown in Figs. 1 and 2, respectively which may be due to the fact the mechanism of action of ACTs involve endoperoxides which lead to the generation of free radicals to kill the malaria parasite, and indirectly leading to a reduction in the antioxidant enzymes compared to the normal control group. This agrees with the findings of Obianime *et al.*[20], Edagha *et al.*[22], Chiagoziem *et al.*[76] and Bhattacharyya *et al.*[78]. Certain active oxygen species generated and accumulated in such red blood cells infected with malaria might in turn kill the parasites[14,73].

There was significant increase of testicular weight in ACT-test groups compared with NC, except in AL and DP-test groups as shown in Table 1, which was possibly associated with inflammation. Infections and inflammation may overwhelm immunosuppressor mechanisms inducing autoimmune reactions against spermatic antigens which results in a spermatogenesis and infertility[79]. Macrophages are known to produce cytokines and other mediators involved in orchestrating inflammatory response[80]. This may be a possible mechanism by which the testicular weights increased[21].

There was a marked increase in testicular volume across the ACT-administered groups compared with NC, except in the ASP-administered group, which had the least volume. The rise or fall in a relative weight or organ size after administration of a chemical agent is a pointer to toxicological effects[76]. Organ swelling, atrophy or hypertrophy may represent adaptations to toxicities[82], as the functional anatomy of the organ becomes affected, and this can be predicted through weight determination[76]. Likewise, the results of testicular length and diameter both showed slight significant increase in ACT-administered groups compared to the control group.

Testosterone concentrations increased across the ACT-administered groups compared to Normal control as shown in Table 2. Significant increase was shown in AP, DP and AM test group respectively. This findings conflict with the findings of Aprioko and Mandkwe,[14] that artesunate-amodiaquine (4/10 or 8/20 mg/kg), artesunate-sulfadoxine-pyrimethamine (4 plus 1.25/25 or 8 plus 2.5/50 mg/kg) and artemether-lumefantrine (4.4/27.2 or 8.8/54.4 mg/kg in two divided doses) decrease testosterone level in guinea pigs but agree with the findings of Samuel *et al.*[83], that artesunate-amodiaquine (2.86/8.58mg/kg) and artemether-lumefantrine (1.14/6.86mg/kg) did not cause decrease in testosterone level in Wistar rats. The process of spermatogenesis requires testosterone, and without adequate volume, men are infertile[14,22,83]. The finding of this study suggest that the presence of BTB in the seminiferous epithelium, plays a role in segregating the events of post-meiotic germ cell development from the systemic circulation and also the increase in testosterone level is a result of a surge in testosterone production before the penetration of ACTs through the BTB[66,84]. Developing spermatids possesses the ability to pump harmful substances outside the cells and to selectively uptake substances necessary for their maturity and development suggesting the increase in testosterone level[66,84,85].

The concentrations of FSH and LH were decreased across the ACT-administered groups compared with normal control as shown in Table 2. The result of this study agrees with Aprioko and Mankwe[14], that decrease in FSH and LH was shown in artesunate-amodiaquine (4/10 or 8/20 mg/kg), artesunate-sulfadoxine-pyrimethamine (4 plus 1.25/25 or 8 plus 2.5/50 mg/kg) and artemether-lumefantrine (4.4/27.2 or 8.8/54.4 mg/kg in two divided doses) administration. This suggests that ACTs-administered groups down regulate the production of FSH and LH through the hypothalamic gonadotropin releasing hormone (GnRH)[14,83]. LH and FSH in males, helps in regulations of testosterone production from Leydig cells, and the testicular growth and production of an androgen-binding protein by the Sertoli cells, which are necessary for sustaining the maturing sperm cell[14,83].

Elevated plasma levels of prostate-specific antigen (PSA > 4 ng/mL); a glycoprotein normally expressed by prostate tissue is the typical biomarker utilized for the detection of benign prostate hyperplaxia (BPH) progression or prostate cancer[86]. In this study, there was decrease in PSA concentrations across the ACT-administered groups compared with the NC, with DP-administered group being the most significantly decreased. It has been reported that 3 alpha hydrosteroid dehydrogenase is an enzyme necessary for the synthesis of endogenous neurosteroids like allopregnanolone and 3 alpha androstanediol[87]. Testosterone reduction to the active form dihydrotestosterone, is catalyzed by the enzyme 5-alpha reductase. An increase in testosterone suggests deactivation, decrease or inhibition of the activity of 5-alpha reductase by the ACTs which favours the BPH or Pca progression indicated by the decreased PSA concentrations across the ACT-administered groups, thus implying a less likelihood for occurrence of BPH

or Pca which corroborates reports that certain drugs, chemicals or herbs can alter the activity of 5-alpha reductase[88].

The decreased sperm count across ACT-administered groups except in the AA-administered group. This shows that the drug was able to cross the blood-testis barrier as Sertoli cells in the testis constitute this membrane[89]. The decreased sperm count implies a decrease in number of viable Sertoli cells as treated with artesunate due its toxicity exposing germ cells and developing spermatocytes, and spermatids within semineferous tubule of testis to the drugs toxic effects, thus causing impairment of male reproduction[90].

There was decrease in sperm motility across the ACT-administered groups as shown in Figure 6. This study support the findings of Anyasor *et al.*[91], that arthemeter (25, 50 and 75 mg kg^{-1} day^{-1}) decrease sperm motility and Apiroko and Mankwe[14], that artesunate-sulfadoxine-pyrimethamine (4 plus 1.25/25 or 8 plus 2.5/50 mg/kg) and artemether-Lumefantrine (4.4/27.2 or 8.8/54.4 mg/kg in two divided doses) also decrease sperm motility. The observed reductions in sperm functions in this study might be due to the alteration in micro-environment of the epididymis[18,91] and also suggesting that ACTs affect maturation of spermatozoa in the epididymis[92]. Decrease in sperm motility and sperm count observed in ACT administered groups suggest that there was increase in number of dead spermatozoa in the epididymis which may be associated with the increase in oxidative stress due to increase in ROS[91,93].

The reducing effect exhibited generally by the drugs on sperm motility states a non- beneficial effect as regarding reproductive efficiency in the rats because when sperm cells are not motile and viable, the rate of fusion with an egg is compromised leading to infertility. This immobility could be as a result of the fact that the bioactive compounds in the drug had caused immobilization or weakening effects on the sperm cell[94]. According to Sharpe[95], chemical actions during the spermatogonial phase will probably have greater effect on sperm output, than the action during the spermatids formation phase. It is also known that sperm cells gain their motility as they move through different regions of the epididymis, their motility increases sharply and continues through the cauda epididymis and vas deferens[96]. Artesunate and arthemeter as a monotherapy destroys *Plasmodium* by the generation of free radicals alkylating the parasite membranes[90,93]. The significant reduction observed on the progressive sperm motility and viability of rats treated with artesunate could also be due to free radicals generating capacity of the drug[14,91]. Free radicals have been implicated in male fertility by decreasing sperm motility. Arrest of sperm motility is definitely due to the chain of reactions induced by reactive oxygen species which can affect sperm axoneme function[14,91].

Sperm morphology showed a marked increase in number of abnormal sperm cues across ACT-administered groups compared to NC. Artemether-lumefantrine at 2.29 mg/kg/day caused reduction in the mean abnormal sperm morphology[18]. Exposure to arthemeter as a monotherapy for three days at a very high dose level 160 mg/kg/day caused impairment to reproductive activity exhibited by sperm count motility and morphology[18]. Most of the ACT-administered groups exhibited azoospermia implying drug acute toxicity[90].

The histological assessments of the ASP, DP and APy-administered groups demonstrated the distortion of germinal epithelium from the basement membrane and visible hypocellularity when compared to the NC group as shown in Fig. 7. This implies that there is reduction of viable sperms leading to possible reduced fertility within and immediately after this therapeutic exposure. This finding agrees with the report of Jewo *et al.*[97,] in which Artesunate administration was found to suppress spermatogenesis. Degenerative changes shown in all ACT-administered groups but severe in ASP, DP and APy-administered groups have been reported to result in cell death; apoptotic or necrotic[98]. In this study, ASP, APy and DP test group may have acted indirectly through the generation of high levels of ROS or directly as toxin to cells of the testis, affecting their cellular integrity and causing defects in membrane permeability and cell volume homeostasis. These histopathological findings are supported by the results of its testicular antioxidants, hormones and seminal assessments in Figs. 1 - 6.

Resistance and toxicity of ACTs can be directly proportional to the elimination half life of the drugs[54], with the half life of anti-malarial drugs ranging from about 45 minutes (artemisinin) to weeks (e.g., 3 - 5 weeks for piperaquine)[54,94], thereby posing threat to the histoarchitecture of the testes in Wistar rats.

In conclusion, oral therapeutic exposures to ACTs elicited mild to severe gonadotoxicity with severity of testiculo-microstructural alterations in the increasing order; AA, AP, AL, AM, APy, DP to ASP, respectively. Alterations in testicular hormonal ranged from mild to severe in the increasing order; AA, AL, AM, APy, DP, AP to ASP, respectively, and also alterations in oxidative markers ranged as follows; AL, AL, AM, AP, DP APy to ASP, respectively while generally decreasing PSA level.

Funding

This study was self-funded by the authors.

Acknowledgements

Authors are grateful to Mr. Collins N. Igajah Endocrine laboratory, Department of Biochemistry, University of Calabar and Mr. Oyedele Ajayi Department of Histopathology, University of Uyo Teaching Hospital for their technical assistance.

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Received: March 25, 2023
Accepted: May 5, 2023

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