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Efficacy of six artemisinin-based combination therapies in the attenuation of Plasmodium berghei-induced testicular toxicity in Swiss mice

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SUMMARY

Many artemisinin-based combination therapies (ACTs) have been approved for malaria treatment, yet reports indicate that some ACTs pose reversible testicular toxicity; however there is no comparative study of these ACTs on the testes in a curative malarial model. We investigated the ameliorative activity of six ACTs on Plasmodium berghei (PB) induced perturbations in testicular antioxidants, serum testosterone levels, sperm motility and the testes microanatomy. Forty male Swiss mice were divided into 8 groups of 5 each: Group 1 normal control (NC), uninfected and untreated, received placebo; group 2 was parasitized non-treated (PNT), while groups 3 - 8 received PB inoculum intraperitoneally. Initial parasitemia was established after 72 hours. Groups 3 - 8 thereafter received oral therapeutic doses of artesunate/ amodiaquine (PBAA), artesunate/mefloquine (PBAM), artesunate/sulfadoxine-pyrimethamine (PBASP), artemisinin-piperaquine (PBAP), dihydroartemisinin/piperaquine (PBDP) and artemether/lumefantrine (PBAL) per kg body weight respectively. Final parasitemia was performed 24 hours after last treatment, and animals euthanized. Result for parasitemia level was significantly ($p < 0.05$) declined in ACT-treated groups, except PBASP compared with PNT. Enzymatic antioxidants were significantly ($p < 0.0001$) altered in ACT-treated groups compared to PNT. Non-enzymatic antioxidants were significantly ($p < 0.0001$) increased in PBDP compared to NC and PNT. Progressive sperm motility significantly ($p < 0.0001$) declined in PNT, PBASP, PBAP and PBDP groups compared to NC. Testosterone showed decreasing trend in PBAP compared to PNT, and severe testicular distortions were demonstrated in PNT, PBASP, PBAP and PBDP. This study concludes that therapeutic doses of AA, AM and AL moderately protects against the deleterious effects of Plasmodium berghei-induced testicular toxicity in Swiss mice.

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INTRODUCTION

Malaria is a life-threatening disease caused by *Plasmodium*, a protozoa transmitted to people through the bite of infected female Anopheles mosquitoes. It is the most prevalent parasitic disease worldwide, recently infecting an estimated 216 million persons globally, with the most cases reported in the African region - 90%, followed by the South-East Asia region - 7% and the Eastern Mediterranean region - 2% (WHO, 2017). In Europe, malaria continues to be exclusively imported, with approximately 11,000 cases reported each year, making it the most frequently imported tropical disease (Ramirez-Olivencia et al., 2012). Severe malaria is associated with multiple organ dysfunctions, and the main cause of death (Ratan et al., 2013). Some of the organ dysfunctions and health conditions associated with malaria include: oliguric acute kidney injury (Vakrani et al., 2016), renal failure, cerebral malaria, jaundice, severe anemia (hemoglobin [Hb] ≤ 5 g/dl), severe thrombocytopenia (abnormally low platelets in the blood), shock, septicemia; a disease caused by presence of bacteria or toxins in the bloodstream characterized by chill and fever (Ratan et al., 2013).

Malaria treatment has evolved substantially over the years (Achan et al., 2011), and currently, 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 complicated malaria,

both in endemic and non-endemic countries (Askling et al., 2012; Munoz et al., 2015; WHO, 2015).

The ACT demonstrates greater efficacy in treating the disease, reducing transmission in endemic areas and producing lower levels of reinfection, (Bouchaud et al., 2012; Sagara et al., 2012; Abay, 2013). ACT is a co-formulation of the main drug; artemisinin or its derivatives, and the companion/ partner drug(s). Artemisinin partner drugs includes: lumefantrine, mefloquine, amodiaquine, sulfadoxine-pyrimethamine, and chlorproguanil/dapsone, while artemisinin derivatives include; dihydroartemisinin, artesunate and artemether (Malaria Consortium, 2018). Artesunate has proven to be superior to quinine in most situations where complicated malaria is treated (Dondorp et al., 2005; Dondorp et al., 2010; Maka et al., 2015).

Testicular histology have revealed deleterious effect of artemisinin derivatives such as varying degree of cell clustering, cellular hypertrophy and intercellular vacuolations specifically in the germinal cell layer resulting in a decline in sperm production (Rajput et al., 2012). ACTs may cause renal and hepatic toxicity (Etim et al., 2016; Silva-Pinto et al., 2017), it affects both enzymatic and non-enzymatic antioxidants (Olayemi et al., 2012). Farombi et al., (2015) concluded that artemisinin-induced mechanisms of uterine and erythrocyte toxicities seem to be associated with the induction of oxidative stress in the tissues: thus an antioxidant is required after the malaria treatment with ACTs to attenuate this effect. A report on long and short-term administration of ACTs (artemether/ lumefantrine and dihydroartemisinin/piperaquine) resulted in a reversible alteration of sperm parameters and reduction of testosterone: this was partly attributed to oxidative stress (Daikwo and Kawa, 2015; Kareem et al., 2015).

The aim of this study is to characterize the effects (biochemical and histological) of 6 ACTs: artesunate/ amodiaquine (AA), artesunate/ mefloquine (AM), artesunate/ sulfadoxine-pyrimethamine (ASP), artemisinin/ piperaquine (AP), dihydroartemisinin/ piperaquine (DP) and artemether/ lumefantrine (AL), on testicular antioxidant levels, testicular microanatomical and testosterone alterations in an experimental malaria murine model.

MATERIALS AND METHODS

Materials

Forty (40) adult male Swiss mice obtained from the Faculty of Basic Medical Sciences Animal House, University of Uyo were used for the study. The animals were allowed to acclimatize for 2 weeks, and given humane care in accordance with the Principle of Laboratory Animal Care and Use (National Research Council, 2011). The animals were housed in well-ventilated wooden cages under controlled environmental conditions of temperature 25 ± 5%, and 12-hour light/dark cycle, and were fed with UAC Vital feed® – a Pelletized Growers Feed (Grand Cereals Ltd, Anambra, Nigeria), and provided water *ad libitum* throughout the experiment.

Experimental design

The adult Swiss male mice were randomly divided into 8 groups of 5 animals each as follows: Group 1 – the normal control (NC) – which were uninfected and untreated; Group 2 served as the parasitized non-treated (PNT); the test Groups 3-8 received Plasmodium berghei (PB) inoculum 1 x 106 via a single intraperitoneal injection. After 72 hours the initial parasitemia was established. Groups 3 - 8 received oral therapeutic doses of artesunate/amodiaquine - (AA [Camosunate® 5.71 mg]), artesunate/mefloquine - (AM [Artequin® 6.43 mg]), artesunate/sulfadoxine-pyrimethamine - (ASP [Simbcure® 25.36 mg then 2.86 mg] for day 1 and 2 respectively), artemisinin/piperaquine -

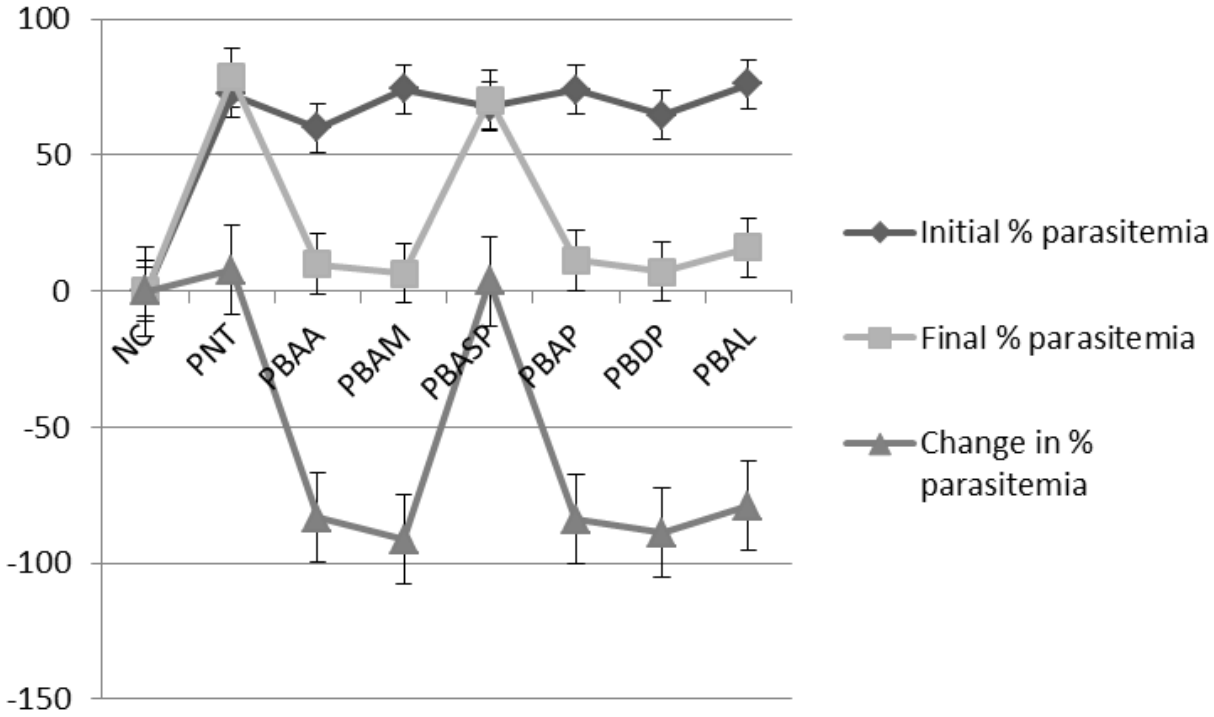

Fig 1. Effect of six ACTs on parasitemia in *P. berghei*-infected Swiss mice.

(AP [Artequick® 12.5 mg]), dihydroartemisinin/ piperaquine (DP [P-alaxin® 5.14 mg]) and artemether/lumefantrine - (AL [Coartem® 8 mg]), per kg body weight of the animal respectively. The final parasitemia were determined 24 hours after the last treatment.

Inoculation of parasite

The parasite *P. berghei ANKA* chloroquine resistant strain was obtained from the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo. A standard inoculum 1×10^6 of parasitized erythrocytes was prepared from the donor mouse in volume of 0.2 ml, to passage the experimental animals via a single intraperitoneal injection (Basir et al., 2012). Infectivity was monitored by thin smear and allowed to progress until 72 hours later during which hyperparasitemia was reached (Rosangelia et al., 2012).

Drugs preparation and dosage

The drugs were prepared separately by mashing each tablet gently in a small crucible with ceramic pestle, and the powder dissolved in 400 ml of distilled water to produce a stock solution. The doses administered were calculated using the formula below;

Dose = weight of animal (g)/ 1000 × dosage/ stock; where dosage = weight of drug (mg)/ 70 kg; and stock = weight of drug (mg)/ volume of distilled water (ml).

The therapeutic doses of the ACTs used were: AA = 400 mg; AM = 1350 mg; ASP = 725 mg; AP = 1000 mg; DP = 360 mg; and AL = 560 mg per kg body weight of the animals respectively. All drugs were administered via the oral route as per the standard regimen of between 2 – 3 days as stipulated by the manufacturers in the drug leaflets.

Animal sacrifice and collection of samples

The animals were euthanized on day 7 by chloroform inhalation, and blood collected via intracardiac puncture. Each testis was excised and separated from the cauda epididymis, then weighed using an electronic balance (Mettler Toledo; Microstep (Pty) Ltd., Greifensee, Switzerland), and an average weight was recorded for the two testes for each animal. One testis from each animal was subsequently fixed in Bouin's fluid for tissue processing and photomicrography.

Relative organ weight

The relative organ weights for the testes = organ weight/ final body weight x 100.

Testicular antioxidant

The testicular antioxidant activities from testes homogenates of Swiss mice were determined for superoxide dismutase (SOD) as described by Sun and Zigma (1978), malondialdehyde (MDA) as described by Buege and Aust (1978), catalase (CAT) as described by Sinha et al., (1971), reduced glutathione (GSH) by Sedlak and Lindsay (1968), glutathione peroxidase (GPx) as described by Rotruck (1973). Glutathione S-transferase (GST) as described by Habig et al., (1974), The vitamins (C, E and A) were determined as described by Omaye et al., (1979), Baker et al.,

Table 1. Testicular weight of control and experimental groups.

Group	Initial Body weight (g)	Final Body weight (g)	% Change in Body weight	Left Testis (g)	Right Testis (g)
NC	26.8	27.4	+2.19	0.08 ± 0.01	0.10 ± 0.01
PNT	21.6	20.4	-5.88	0.08 ± 0.01	0.08 ± 0.01
PBAA	24.0	22.8	-5.00	0.06 ± 0.01	0.06 ± 0.02
PBAM	25.0	22.2	-12.73	0.08 ± 0.01	0.08 ± 0.01
PBASP	27.6	26.4	-4.55	0.09 ± 0.00	0.08 ± 0.01
PBAP	32.4	30.2	-7.28	0.08 ± 0.01	0.08 ± 0.01
PBDP	28.0	26.2	-6.87	0.08 ± 0.00	0.08 ± 0.01
PBAL	26.0	24.0	-8.33	0.08 ± 0.01	0.09 ± 0.01

Values are expressed as Mean ± SEM; $p < 0.05$, NC – normal control, PNT – Parasitized non-treated, PBAA – *P. berghei* infected then artesunate/ amodiaquine treated, PBAM – *P. berghei* infected then artesunate/mefloquine treated, PBASP – *P. berghei* infected then artesunate/ sulfadoxinepyrimethamine treated, PBAP – *P. berghei* infected then artemether/ piperaquine treated, PBDP – *P. berghei* infected then dihydroartemisinin/ piperaquine treated, PBAL – *P. berghei* infected then artemether/ lumefantrine treated.

(1980) and Rutkowski et al., (2006) respectively.

Testosterone assay

The serum levels of testosterone was determined using Agappe® enzyme immunoassay (ELISA) kit with catalog number 3725-300A for free testosterone, in accordance with manufacturer's protocol.

Semen collection and analysis: The testes were excised from the scrotum of the mice across all the groups, together with the epididymis, and weighed using Mettler Toledo analytical balance (Microsep (pty) Ltd., Switzerland). Thereafter all the epididymides were removed from the testis for semen fluid analysis. The semen was obtained from the caudal epididymis and placed in a petri-dish which contained 5 mL of normal saline. A drop of the semen mixed with normal saline was put on slide, cover-slipped and viewed under the light microscope. The motility of 100 spermatozoa was randomly assessed, and categorized as motile; progressive or non-progressive, and immotile.

Histological assessment: The testes were dissected and fixed in Bouin's fluid for 6 hours and transferred to 70% ethanol (Latendresse et al., 2002). Testicular tissue samples were then processed using graded series of ethanol and paraffin -embedded. The paraffinized wax tissue blocks were sectioned at 5 µm using the rotary microtome - Thermo Scientific (MicromHM 325, Germany), then the gelatinized tissue slides were stained with haematoxylin and eosin (H&E) for general testicular morphology (Cardiff et al., 2008). The photomicrographs of tissue sections were obtained with Amscope digital camera (MU 1000, China) coupled to a light microscope (Olympus - CX31 Tokyo, Japan).

Statistical analysis

Data obtained from the study were analyzed and expressed as mean ± standard error of mean using the Graphpad 6 version 11 system packages. One-way ANOVA, multiple comparison were employed to determine the level significance at ($p < 0.05$).

RESULTS

Effect of the six ACTs on parasitemia

The percentage parasites clearance were significantly ($p < 0.05$) reversed in all ACT-treated groups, except PBASP group compared to PNT (Fig. 1).

Effect of the six ACTs on the testicular histology

The morphological alterations in the testes following the administration of 6 ACTs in a *Plasmodium*-induced testicular toxicity are presented in Fig. 2. The H&E stain demonstrated good morphology in the seminiferous tubules of PBAA-, PBAM- and PBAL-treated groups comparable with NC. However, PBASP-, PBAP- and PBDP-treated groups showed severe distortions of the spermatogenic lineage cells, reduction of spermatozoa in the lumen of the seminiferous tubules and mild degeneration of the interstitium, comparable with the PNT group.

Effect of the six ACTs on body and testicular weights

All parasitized groups had a net negative body weight compared to NC. There was no significant change in the weight of the testes in PNT, PBAM, PBAL, PBAP and PBDP groups compared to NC. However, there was significant ($p < 0.05$) decrease in the weight of left testes in PBAA, and a significant ($p < 0.05$) increase in the weight of left testes in PBASP compared to NC. The right testis showed decreased weight in all the treatment groups compared to NC, although significant (p <

Table 2. Effect of six ACTs on testicular enzymatic antioxidants.

Group	SOD (µmol/ml/ min/mg pro)	MDA (µmol/ ml)	CAT (µmol/ml/min/mg pro)	GSH (µmol/ml)	GPx (µmol/ml/min/ mg pro)	GST (µmol/ml/ min)
NC	12.77 ± 0.31	2.84 ± 0.44	100.46 ± 4.74	18.26 ± 0.98	3.77 ± 0.09	24.28 ± 1.30
PNT	10.69 ± 0.59	2.38 ± 0.03	77.86 ± 3.33	25.83 ± 2.68^{c}	3.19 ± 0.18	35.06 ± 3.33
PBAA	9.16 ± 0.22	2.88 ± 0.24	78.21 ± 2.35	24.84 ± 0.93	2.70 ± 0.07	30.03 ± 1.91
PBAM	12.58 ± 0.85	3.59 ± 0.49	127.91 ± 4.66	11.44 ± 0.82^{d}	3.82 ± 0.29	14.08 ± 1.11^{f}
PBASP	14.86 ± 1.63	6.37 ± 0.86^{a}	166.30 ± 19.53^{b}	25.04 ± 1.92	4.46 ± 0.52^{e}	37. 67 ± 4.99
PBAP	22.58 ± 2.27***	3.00 ± 0.20	229.69 ± 23.33***	19.09 ± 0.36	6.76 ± 0.66***	33.02 ± 0.62
PBDP	20.82 ± 1.52***	4.46 ± 0.67	211.82 ± 17.63***	24.17 ± 3.21	5.81 ± 0.42***	36.75 ± 5.77
PBAL	16.32 ± 0.90	5.53 ± 0.88^{a}	131.50 ± 8.37^{b}	10.45 ± 0.61^{d}	5.02 ± 0.28^{e}	12.26 ± 0.62^{f}
P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Values are expressed as Mean ± SEM; p < 0.05, SOD – superoxide dismutase, MDA – malondialdehyde, CAT – catalase, GSH – glutathione, GPx – glutathione peroxidase, GST – glutathione S-transferase, *** - Significantly increased compared to other test groups and NC. *** - Significantly increased compared to other test groups and NC; a - Significantly increased compared to other test groups and NC except PbDP; b - statistically increased compared to NC, PNT and PbAA; c - Significantly increased compared to NC, PbAM, and PbAL; d - Significantly decreased compared to NC, PbAA, PbAP and PbDP; e - Significantly increased compared to PNT and PbAA; f - Significantly decreased compared to NC and other test groups.

0.05) decrease was observed in the PBAA group (Table 1).

Effect of the six ACTs on testicular enzymatic antioxidants

The SOD was significantly (p < 0.05) increased in NC and other treated groups except PBAA which had decreased SOD compared to PNT. However, PBDP, PBAP, PBAL, and PBASP showed increased SOD compared to NC (Table 2).

The MDA was significantly increased (p < 0.05) in PBAA, PBAM, PBASP, PBAL, PBAP and PBDP compared to PNT. The PNT showed decreased MDA compared to NC (Table 2).

The CAT significantly (p < 0.05) decreased in PNT and PBAA groups compared to NC. However, CAT was significantly (p < 0.05) increased in PBAM, PBASP, PBAL, PBAP and PBDP groups compared to NC, while all treated groups showed elevated CAT compared to PNT.

Reduced glutathione (GSH) decreased in all treated groups compared to PNT, in the order PBAA > PBDP > PBASP > PBAP > PBAM > PBAL. However, significant (p < 0.05) increase in GSH was observed in PBAA, PBASP, PBAP and PBDP groups compared the NC (Table 2).

Glutathione peroxidase (GPx) showed significant (p < 0.05) increase in the order PBDP > PBAL > PBASP > PBAM > NC compared to PNT. However, significantly (p < 0.05) decreased GPx was observed in the PBAA (Table 2).

Glutathione S-transferase (GST) significantly (p < 0.05) decreased in PBAP, PBASP, PBAL, PBAM

Table 3. Effect of six ACTs on testicular non-enzymatic antioxidants.

Group	Vit. A (µg/100g)	Vit. C (mg/g)	Vit. E (µg/100g)
NC	314.32 ± 21.22	19.03 ± 1.98	160.23 ± 26.68
PNT	1219.51 ± 186.54	32. 43 ± 2.70^{b}	430.30 ± 56.42
PBAA	666.59 ± 69.53^{a}	29.21 ± 2.21^{b}	1067.05 ± 39.25
PBAM	546.59 ± 32.28	26.28 ± 1.67	1016.29 ± 61.44^{d}
PBASP	715.38 ± 68.59^{a}	25.04 ± 1.25	1474.61 ± 175.60^{e}
PBAP	298.49 ± 40.36	10.37 ± 1.26^{c}	421.59 ± 95.40
PBDP	1084.73 ± 102.42***	38.97 ± 3.14***	1640.53 ± 177.33***
PBAL	602.05 ± 44.97	25.76 ± 1.53	679.17 ± 98.11^{f}
P value	0.0001	0.0001	0.0001

Values are expressed as Mean ± SEM; p < 0.05, ***- significantly increased compared to NC and other test groups; a - significantly increased compared to NC and PBAP; b - significantly increased compared to NC and PBAP; c - significantly decreased compared to all test groups and NC; d - significantly increased compared to NC, PBAA, PBAP, PBAL; e - significantly increased compared to all test groups except PBDP; f - significantly increased compared to NC.

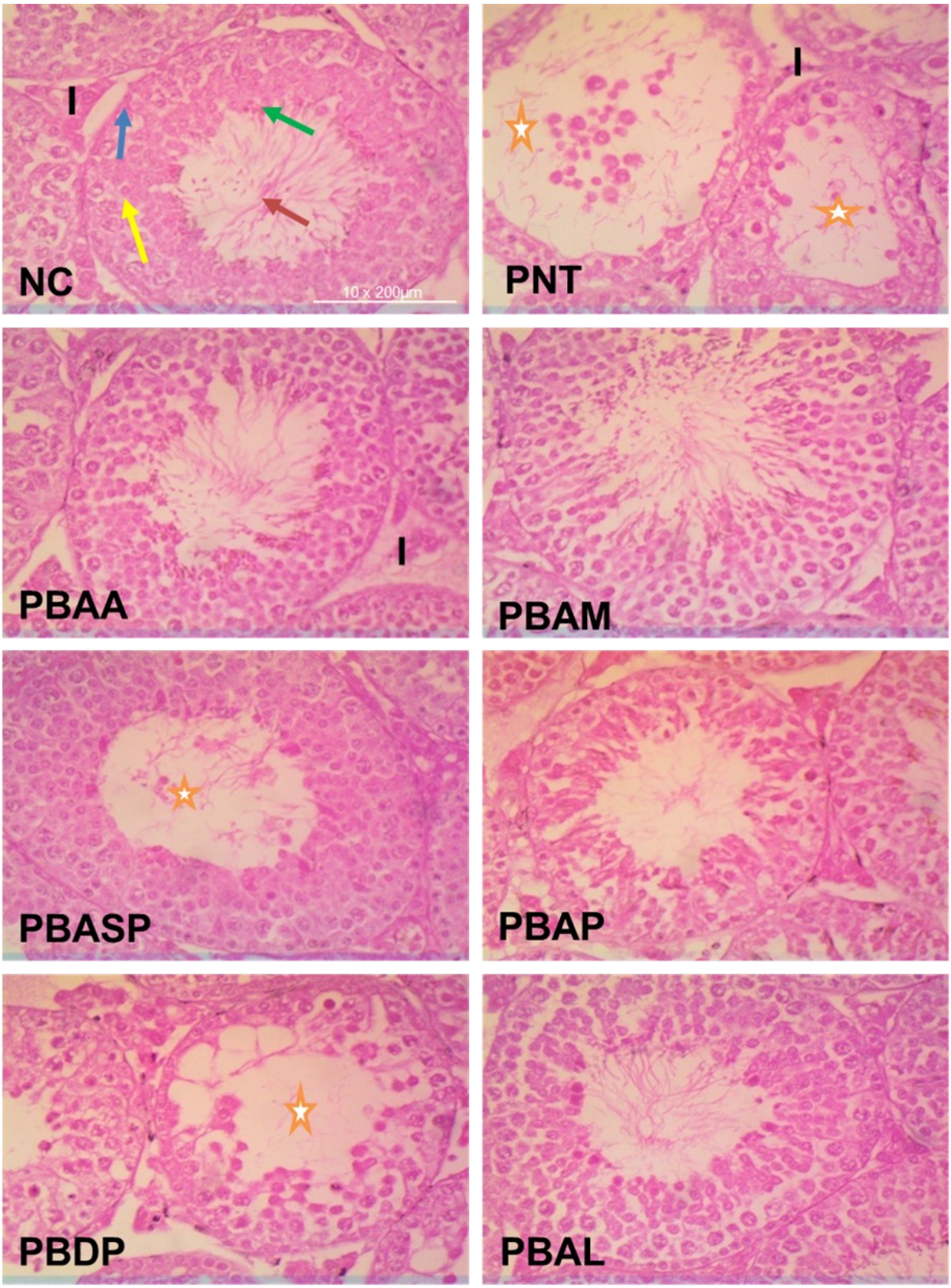

Fig 2. The representative of H&E stained photomicrographs of testicular tissues. Normal testes of uninfected and untreated (NC) show normal histological structure of seminiferous tubules (ST) with active functioning and complete spermatogenic series, and (I) interstitial space. Cells at the peripheral layer are composed of spermatogonia (blue arrow) and spermatocytes (yellow arrow), then a zone of spermatids (green arrow) and mature spermatids (green arrow). Mice testes of parasitized non-treated (PNT) show the presence of degenerated spermatogenic series in tubular lumen (red arrow). The ST in PBAA, PBAM, and PBAL were similar to NC group. Note the atrophy of seminiferous tubules starred (*).

and PBAP treated groups compared to PNT; however, PBDP showed increased GST. The PBDP, PBAP, PBASP, and PBAA treated groups had elevated GST, whereas PBAM and PBAL showed decreased GST compared to NC (Table 2).

Effect of the six ACTs on testicular non-enzymatic antioxidants

Vitamin C significantly ($p < 0.05$) decreased in NC, PBAA, PBAM, PBAL, PBASP, and PBAP groups, and increased in PBDP compared to PNT. However Vitamin C significantly ($p < 0.05$) decreased in PBAP, but significantly ($p < 0.05$) increased in PBAA, PBAM, PBASP, PBAL and PBDP groups compared to NC (Table 3).

Vitamin E significantly ($p < 0.05$) increased in PBDP, PBASP, PBAA, PBAM, and PBAL groups, but decreased in PBAP compared to PNT. The ACT treated groups showed significant ($p < 0.05$) increase in the levels of vitamin E compared to the NC (Table 3).

Vitamin A significantly ($p < 0.05$) decreased in the ACT-treated groups compared to the PNT and increased in treated groups except PBAP compared to the NC (Table 3).

Effect of the six ACTs on serum testosterone levels

The level of testosterone was significantly ($p<0.05$) reduced in PBAP compared to PBAA, PBAM and PBASP treated groups (Table 4).

Effect of the six ACTs on sperm motility levels

The NC and PBAL had significantly ($p < 0.001$) increased progressive motility compared to all other test groups, PBAA and PBAM also had significantly ($p < 0.0001$) increased progressive motility compared to PNT, PBASP, PBAP and PBDP. Non-progressive motility were significantly ($p < 0.0001$) increased in PNT, PBASP, PBAP and PBDP groups compared to NC, PBAA, PBAM and PBAL groups. The immotile sperms were significantly higher in PNT, PBASP and PBAP compared to NC and other groups, likewise NC and PBAL had significantly ($p<0.0001$) lower immotile sperms compared PBAA, PBAM and PBDP.

Table 4. Effect of six ACTs on serum testosterone concentration.

Group	Testosterone (ng/ml)
NC	15.39 ± 0.60
PNT	15.64 ± 0.35
PBAA	15.75 ± 0.51
PBAM	15.97 ± 0.11
PBASP	15.83 ± 0.40
PBAP	13. 94 ± 0.12***
PBDP	15.23 ± 0.08
PBAL	15.14 ± 0.16
P value	0.007

Values are expressed as Mean ± SEM; $p < 0.05$. *** - significantly decreased compared to PBAA, PBAM and PBASP groups.

DISCUSSION

The therapeutic doses of ACTs used in this study generally interrupted parasite multiplication and caused parasite clearance as earlier reported (Onyamboko et al., 2014), except ASP, which was ineffective in parasite clearance (Maiga et al., 2015). ACTs have been reported to cause variable effects on testicular parameters which imply testicular toxicity (Raji et al., 2005), mediated by the artemisinin agent (Nwanjo et al., 2007).

The result of testicular weight showed decrease in both the right and left testes with decrease in PBAA, and an increase in weight of left testis of PBASP compared to NC. There was a decrease in the weight of right testis in treated groups and PNT compared to NC. Increase or decrease in relative or absolute weight of an organ after introduction of a chemical agent is an indication of toxic effect (Simons et al., 1995; Maina et al., 2008). Toxicity may manifest as swelling of organ, atrophy or hypertrophy (Afolayan and Yakubu, 2009), thus the functional anatomy of the organ could be altered and this can be assessed experimentally through its weight (Simons et al., 1995; Raji et al., 2005).

Seminal plasma contains antioxidants enzymes such as superoxide dismutase, glutathione peroxidase, catalase, malondialdehyde (Alvarez et al., 1987; Pons et al., 2003; Ishii et al., 2005), and antioxidant non-enzymes such as vitamins A, C and E (Kutlubay et al., 2007; Colagar et al., 2009). Normal sperm functions include acrosomal reaction, sperm capacitation and sperm-oocyte fusion which require a balance between the antioxidants and oxidants (Sikka, 2001). Excess oxidants in the testis may overpower the defense mechanism of antioxidants hence causing oxidative stress which causes damages to spermatozoa thereby compromising sperm quality and functions (Sikka, 2001).

Testicular MDA levels in this study showed a significant increase in ACT-treated groups compared to NC and PNT in the order; PBAL > PBASP > PBDP > PBAM > PBAP > PBAA. This result corresponds with report on increased testicular MDA level observed in rats treated with artemether lumefantrine (Daikwo et al., 2011; Daikwo et al., 2018), thus PBAL raised MDA levels.

Increased SOD in the PBAL group conflicts with the findings of Daikwo et al., (2018), that prolonged administration of AL decreased SOD level in rats. The ACTs-induced increase in antioxidant enzymes could be due to their oxidant activity which elevated the enzymatic antioxidant levels as part of the defense mechanism of the body (Ukwenya et al., 2010).

The PNT showed decreased testicular SOD,

Table 5. Effect of six ACTs on sperm motility.

Group	% Progressive motility	% Non-progressive motility	% Immotile
NC	74 ± 0.93***	15.80 ± 1.59	9.60 ± 0.93
PNT	16.20 ± 1.16	45.00 ± 1.41[c]	38.80 ± 2.27[d]
PBAA	59.40 ± 2.50[a]	19.80 ± 2.78	20.80 ± 1.46[e]
PBAM	55.40 ± 1.69[a]	23.60 ± 1.21	21.00 ± 1.30[e]
PBASP	17.20 ± 2.35	44.60 ± 3.87[c]	38.20 ± 4.28[d]
PBAP	26.40 ± 4.92[b]	36.20 ± 4.71[c]	37.40 ± 3.70[d]
PBDP	30.40 ± 0.81[b]	41.20 ± 1.16[c]	28.40 ± 1.12[e]
PBAL	72.60 ± 1.50***	14.80 ± 0.80	12.60 ± 0.93
P value	0.0001	0.0001	0.0001

Values are expressed as Mean ± SEM; $p < 0.05$, NC – normal control, PNT – Parasitized non-treated, PBAA – *P. berghei* infected then artesunate/ amodiaquine treated, PBAM – *P. berghei* infected then artesunate/mefloquine treated, PBASP – *P. berghei* infected then artesunate/ sulfadoxinepyrimethamine treated, PBAP – *P. berghei* infected then artemether/ piperaquine treated, PBDP – *P. berghei* infected then dihydroartemisinin/ piperaquine treated, PBAL – *P. berghei* infected then artemether/ lumefantrine treated.

MDA, CAT, and GPx levels compared to NC and treated groups except PBAA. These antioxidant reductions could be due to sustained elevated parasite-induced oxidant levels, which overwhelmed the antioxidant enzymes which were used up to conjugate the oxidants, and/or parasite-induced ROS such as highly reactive hydroxyl and peroxynitrite, which may react adversely with the antioxidant enzymes culminating in substrate inhibition, and thus depleting the testicular antioxidant levels (Ukwenya et al., 2010; Nnodim et al., 2012; Olukemi et al., 2018). Hence, the *Plasmodium* parasites adopt this oxidative property to induce oxidative stress and possibly cause testicular inflammation as evident in the histology of the testes (Fig. 2 [PNT]).

The PBDP and PBAP groups were consistent in inducing increased antioxidant enzymes owing to the fact that these enzymes were sensitive to high levels of ROS generated by the artemisinin derivatives or the partner Piperaquine, but PBAM and PBAA stimulated very weak enzymatic antioxidant response probably due to the artesunate/partner drug mediated-ROS interaction which might have mimicked the mechanism of enzymatic antioxidant evasion/attack by parasite-induced ROS against the *Plasmodium* parasites accounting for their efficacy in parasite clearance (Ukwenya et al., 2010), though associated with testicular oxidative stress.

However, PNT showed increased GSH and GST levels compared to treated groups and NC probably indicating that GSH and GST were sensitive to the parasite induced ROS such as hydroxyls, which evaded other antioxidant enzymes (Nnodim et al., 2012). Testicular GST and GSH showed similar trends in the NC, PNT and treated groups, possibly because GST catalyzes the attack by GSH on xenobionts, and hence they operate synergistically and complementarily (Hayes et al., 2005).

Vitamin A was significantly decreased in ACT-treated groups except PBDP and NC compared to PNT. This result corresponds with a report by WHO (1995), that vitamin A level increased in diseased or infectious state and when exposed to xenobionts. This suggests that the ACTs might have mitigated the parasite-induced elevation of vitamin A by reducing parasite-induced oxidant levels via effective parasite clearance. However, PBAP group showed decreased vitamin A compared to NC, and Vitamin A has been found to play a significant role via the testosterone signaling pathway in an active form known as retinoic acid (RA) by stimulating the Leydig cells to respond to luteinizing hormone (LH), and synthesize the male steroid hormone known as testosterone, which plays a meiotic role in spermatogenesis (WHO, 1995; Cathryn et al., 2010), and its alteration or deficiency has been reported to lead to defective spermatogenesis in rodents (Livera et al., 2002; Zhou et al., 2008). Atrophy of spermatogenic cells, degeneration of interstitial tissues and eruption of basement membrane, clearly seen in the histological sections of PNT (Fig. 2) and PBASP groups, could be due to an interruption in spermatogenesis caused by alteration in vitamin A.

Vitamin C supports spermatogenesis by reducing vitamin E and maintaining it in an active state, while it is maintained in a reduced state by a GSH-dependent dehydroascorbate reductase which is abundant in the testes (Paolicchi et al., 1996). The vitamin C significantly increased in treated groups except PBAP, but PBDP had the highest vitamin C level. Falciparum malarial infections in humans have been reported to significantly reduced serum vitamin C in children between the ages of 5 – 15. However, increased levels of this antioxidant were observed in falciparum malarial infected adults. This was concluded to be part of early response to the infection in adult (Uzuegbu, 2011). Previous findings reported that endogenous vitamin C levels

reduced when oxidative stress is induced on testes (Aruldhas et al., 2005). The significantly increased testicular vitamin C seen in PNT and treated groups compared to the NC, could be due to innate homeostatic mechanism by testicular tissues to combat or reduce ACTs/ parasite-induced ROS, hence mitigating oxidative stress, and this might have consequently attenuated serum levels of the Vitamin as previously reported (Aruldhas et al., 2005). Also, a decreased vitamin C level in all treated groups except PBAP treated group compared to PNT was observed.

This study showed that vitamin E level was significantly high in the PNT and treated groups except PBAP. This is likely due to high demand of the vitamin by the testicular tissues to combat or neutralize oxidants produced by the parasites and ACTs as in vitamin C, hence resulting in reduced levels of serum vitamin E (Aruldhas et al., 2005).

Testosterone is produced by the Leydig cells and is required for production of spermatozoa (Aprioku, 2013). The result showed that serum testosterone had slight increases in PBAA, PBAM and PBASP treated groups compared to the PNT and NC owing to the fact that artesunate combination had favorable oxidant influence on the vitamins, and as such the high vitamin level was proportionate to the high testosterone levels. However, PBASP and PBAA decreased serum testosterone in a 14 day study in non-parasitized animal model (Aprioku and Mankwe, 2018). The result also showed slight decrease of serum testosterone in PBAL and PBDP groups, and marked decrease in PBAP group. The slight decrease in PBAL group agrees with previous reports that, AL decreases serum testosterone in non-parasitized animal models (Jimmy and Mbee, 2014; Daikwo and Kawu, 2015; Aprioku and Mankwe, 2018). This result suggests that, ACTs have different effects on the steroidogenic functions of the testes. The increase in serum testosterone in the PNT and some treated groups (PBAA, PBAM and PBASP) compared to the NC suggests that, these ACTs did not affect Leydig cells which secretes testosterone, but might have affected spermatogenic cells that should have utilized the testosterone for spermatogenesis. The decrease in serum testosterone of the other groups (PBAL, PBAP and PBDP) compared to the NC on the other hand suggests that, these ACTs might have affected some Leydig cells, hence, reduction in the production of testosterone. Furthermore, the parasite invasion had no mitigating effect on the testosterone owing to the fact that the vitamins where were sensitive to its ROS, and increased vitamin stimulated increased testosterone.

The generally increased sensitivity of non-enzymatic antioxidants (vitamins A, C and E) to parasite-induced ROS shows that the body utilizes these micronutrients as the chief antioxidant to mitigate oxidative stress that would have been induced by the ROS prior to evasion or subjugation of the enzymatic antioxidant system (Cathryn et al., 2010). On the other hand, PBAP treated group showed a general decline in the testicular vitamins, and this presumably caused the marked decrease in testosterone when compared with other experimental groups, as vitamins play a vital role in the synthesis and secretion of testosterone. The high oxidative tendency of this drug due to its reactive component drugs (Staines, 2012) may induce oxidative stress, which will affect the structural integrity/cellular morphology of spermatozoa, and this corresponds with the histological findings (Fig 2).

Sperm motility can be impaired by toxic substances (Manna et al., 2008), and in this study non -progressive sperm significantly increased in the treated groups compared to PNT corroborated by the histological observations (Fig. 2).

Previous finding indicates that clinical dose of AA caused poor differentiation of sperm cells and damaged the seminiferous epithelium in a non-parasitized in vivo model (Obianime and Aprioku, 2009). Treated groups PBAA, PBAM and PBAL showed better presentations of the spermatogenic cells compared to the PNT. It was however noted that some degree of atrophy was observed in the PBDP, as well as PBASP and PBAP, which may support the findings of Jimmy and Mbee, (2014) that ACTs induces testicular toxicity. This suggests that while some ACTs are testiculo-toxic, others possess some level of testicular protective tendency toward parasite-induced toxicity, or are less toxic. It has been reported that ACTs cause deleterious effect on the testicular tissue, and these effects are reversible (Aprioku, 2013; Aprioku and Mankwe, 2018).

CONCLUSION

This study establishes that AA, AM and AL strongly protected against P. berghei-induced testicular toxicity in Swiss mice following oral therapeutic doses following a 2-3 days standard regimen, whereas ASP, AP and DP mildly attenuated the testicular damage.

AUTHORS' CONTRIBUTIONS

I.A.E, A.U.E, E.I.E, and O.B.O designed the study, collected, analyzed/interpreted data, and drafted the manuscript with O.O.A., A.I.P. Seminal analyses were performed and analyzed by I.E.A and O.B.O. All authors approved the final manuscript.

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