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Comparative Testicular Histopathological Effects of Artemisinin Derivatives and Some ACTs in the Guinea-pig

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ABSTRACT

Artesunate and dihydroartemisinin are artemisinin derivatives, which are effective antimalarial agents used in the treatment of malaria. Combination of artemisinins and other standard antimalarial drugs (ACTs) have resulted in better cure rates of Plasmodium infections. In this study, the histolopathological effects of half, normal and double clinical doses of artesunate, dihydroartemisinin, artesunate/amodiaquine, artesunate/sulfadoxine/pyrimethamine and artemether/lumefantrine on the testes of guinea-pigs were investigated. Mean testicular weight and circumference were both significantly ($p<0.05$) decreased dose-dependently by artesunate/amodiaquine and artemether/lumefantrine, while the other agents decreased only testicular circumference without significant effects on the weight. Furthermore, artesunate and dihydroartemisinin caused dose-dependent distortions of the testicular architecture/histology, characterized by interstitial edema, poor sperm cell differentiation, moderate maturation arrest and impairment of spermatogenesis. The ACTs also caused general inflammation of the testes, with different degrees of edema and depressed sperm production. The clinical dose of artesunate/amodiaquine caused poor differentiation of sperm cells and damage of the seminiferous epithelium. In addition, the half clinical dose of artemether/lumefantrine caused poor development of germ cells, marked maturation arrest and reduced sperm production. There was also marked eosinophilia of the cytoplasm of the spermatocytes, shrinking of the nuclei of the spermatocytes and early signs of necrosis. The results obtained in this study may be due to oxidative damage on the testicular cells/tissues by the antimalarial agents, especially on the Leydig and Sertoli cells of the testis.

Keywords: Artemisinin, ACT, Sertoli, Spermatogenesis, Histopathology

Effective treatment of malaria has been a major health problem, particularly in sub-Saharan Africa. The mosquito-borne, protozoal disease is older than recorded history, and probably plagued prehistoric man (Cowman 1997). Despite enormous and diverse efforts to control this disease, malaria is among the top three most deadly communicable diseases and the most deadly tropical parasitic disease (WHO 1998, Sachs and Malaney 2002). The consequences of the disease are further compounded by extremely low living conditions in some of these poorest nations.

Several drugs have been developed and utilized for the treatment of malaria ranging from the quinine-rich bark of the Cinchona tree (Cowman, 1997) to synthetic compounds including 4-aminoquinolones (e.g. chloroquine, amodiaquine), sulfonamides (e.g, sulfadoxine) antimetabolites (e.g. pyrimethamine), sesquiterpenes (e.g. halofantrine) e.t.c. (Heppner and Ballou 1998, WHO 2000; Katzung 2004). Among these, chloroquine has been the mainstay of malaria chemotherapy for nearly 60 years, but widespread resistance now limits its usefulness (Ridley 1998, Sidhu et al 2002, Sharma 2005). Generally, resistance to most of the drugs by the parasite is a growing problem in the battle against malaria (Markwalder and Meyer, 1982; Zalis et al., 1998, Hoskins et al 1998, Basco et al 1998**)**. The present choices of drugs are artemisinin and its derivatives (White 1997, Haynes 2001).

Artesunate and dihydroartemisinin are artemisinin derivatives which have high killing rate of the plasmodium parasite (White 1997). Furthermore, artemisinin-based combination therapies (ACTs) have been shown to increase the efficacy of the combinants, resulting in better cure rates and clearance of the malaria parasite from the blood (Toure and Oduola 2004, Nosten and White 2007). These combinations include: artesunate/amodiaquine, artesunate/ sulfadoxine/pyrimethamine, artesunate/ mefloquine and artemether/ lumefantrine (Nosten and White, 2007).

Studies had shown that most antimalarial agents cause male reproductive dysfunction in laboratory animals (Joshi et al 1996, Adeeko and Dada 1998, Parveen et al 2003). Semen parameters in mice and albino rats have been reported to be adversely affected by chloroquine and quinine (Adeeko and Dada 1998), halofantrine (Orisakwe et al 2003) and artemsinins (Nwanjo et al 2007). However, most of such studies were carried out with mice or rats. Secondly, though combination of antimalarial agents result in potentiation of the antimalarial effects of the partner drugs (White and Olliaro 1996, Durrani et al 2005), a comparative toxicological effects of ACTs and their partner agents in male reproductive function have not been reported. It is on this note that, in furtherance of our earlier studies of the effects of artesunate and some ACTs on biochemical and spermatic parameters in the male guinea-pig (Obianime and Aprioku, 2009a, Obianime and Aprioku, 2009b), we decided to investigate the testicular histopathological effects of artemisinin derivatives and ACTs in the guinea-pig.

MATERIALS AND METHODS

All the drugs used in this study were obtained from the University of Port Harcourt Teaching Hospital (UPTH), Port Harcourt, Nigeria. Artesunate (Arinate), manufactured by ERFA, Brussels; dihydroartemisinin (Alaxin) manufactured by GVS Labs, India); artesunate/sulphadoxine/ pyrimethamine (Farenax) and artesunate/amodiaquine (Dart) manufactured by Swiss Pharma Nigeria Ltd, Nigeria and artemether/lumefantrine (Coartem) by Norvatis Pharmaceuticals Corporation, Suffern, New York, USA. The agents were dissolved in distilled water and administered oral gavage.

Animals

All animals used in this study were handled in accordance with the international, national and institutional guidelines for Care and Use of Laboratory Animals in Biomedical Research as promulgated by the Canadian Council of Animal Care (1984).

Outbred strains of adult male guinea-pigs (*Cavia porcellus*) of average weight 450±5g were obtained from the animal house of the University of Port Harcourt, Nigeria and allowed to acclimatize for 14 days. They were housed in shoebox cages with wire bar lids used to hold the water bottle and feed to prevent contamination with urine or faeces. Bedding was placed directly into the shoe box cage allowing the absorption of urine. The guinea-pigs were kept in a well ventilated room at ambient temperature of 28.0±2.0 ^{o}C under natural light condition and fed ad litituary with vital layer feeds produced by Topfeeds Ltd, Sapele, Delta State, Nigeria. Water was given *ad libitum*. Generally, the study was conducted in accordance with the recommendation from the declarations of Helsinki on guiding principles in care and use of animals.

Protocol

Experimental animals were divided into 4 groups- A, B, C and D, identified as normal clinical dose, half clinical dose, double clinical dose and control groups respectively. Animals in each group except the control were further subdivided into 5 groups of five animals each. Animals in group A were given 2mg/kg of artesunate 12-h for 3 days; 2.2 mg/kg of dihydroartemisinin on the first day (0 h), followed by 1.1 mg/kg on the next six consecutive days (24 h, 48 h, 72 h, 96 h, 120 h and 144 h); 4 mg/kg of artesunate plus 10mg mg/kg of amodiaquine daily for 3 days; a single dose of 1.25/25 mg/kg of sulfadoxine/pyrimethamine plus 4 mg/kg of artesunate, given daily for 3 days; and 2.2/13.6 mg/kg of artemether/lumefantrine at 0, 8, 24, 36, 48, and 60 h. These were all normal clinical doses of the agents (van Vugt et al 1999, Barnes et al 2006, Nosten and White 2007). Group B received half of the concentrations of the above drugs, while group C had double amounts of the above drugs. Group D was given only distilled water for seven days. Animals were sacrificed

at the end of each treatment course under pentobarbital anesthesia, 37mg/kg IP (Flecknell 1996). The testis was carefully isolated, weighed, washed in buffered saline and fixed in 10% formalin. Testis sections (56 µm) were routinely processed by standard histological techniques, stained with hematoxylin and eosin (H&E), and examined by light microscope (Nikon Eclipse E400) to assess histopathological changes.

Statistical Analysis

Data were expressed as means ± standard errors of mean. Comparisons between control data and treated groups of guinea-pigs were performed with one-way analysis of variance (ANOVA).

RESULTS

Weight and Circumference of testis

Dihydroartemisinin (Alaxin), artesunate (Arinate), artesunate/amodiaquine (Dart), artesunate/lumefantrine (Coartem) and artesunate/sulfadoxine/pyrimethamine (Farenax) caused significant ($p<0.05$) decreases in the testicular circumference. This occurred mainly at the half clinical dose in artesunate and at the double clinical doses in the other agents (Tables 1a and b). Furthermore, artesunate/amodiaquine and artemether/lumefantrine caused significant dose-dependent decreases in weight of the testis, while artesunate, dihydroartemisinin and artesunate/sulfadoxine/pyrimethamine had no significant effect on testicular weight (Tables 1a and b).

Histopathology Result

Dihydroartemisinin (Alaxin) caused distortion of the testicular architecture characterized by inflammation of testicular tissues with destruction of basal membrane (Figs. 1a, b, c and d). There was also vacuolation, poor differentiation of sperm cells, maturation arrest, reduced sperm production and atrophy at the clinical dose (Fig. 1c). Also, artesunate caused inflammation of the interstitial cells and seminiferous tubules. It also caused poor differentiation of sperm germ cells and mild maturation arrest. These effects were dose-dependent (Figs. 2 a, b, c and d).

Furthermore, artesunate/sulfadoxine /pyrimethamine (Farenax) caused dose-dependent inflammation and alterations of the seminiferous epithelium (Figs. 3a, b, c and d). The double clinical dose caused pronounced interstitial edema and destruction of basement membrane of the seminiferous epithelium (Fig. 3d). Artesunate/amodiaquine (Dart) also caused inflammation of the testicular cells, atrophy of primordial sperm cells, moderate maturation arrest and depressed spermatogenesis (Figs. 4a, b, c and d). Artemether/lumefantrine (Coartem) caused marked edema, poor development of germ cells, maturation arrest and impairment of spermatogenesis (Figs. 5a, b, c and d). Furthermore, shrinking of the nuclei of the spermatocytes and early signs of cell necrosis were observed at the half clinical dose, showing that lower concentrations of artemether/lumefantrine may have greater level of toxicity on the testicular histology (Fig. 5b).

Table 1: The effects of different doses of (a) artesunate and dihydrartemisinin and (b) ACTs on the testicular weight and circumference of the male guinea-pig. Data are means ±SEM. * statistically significant at $p<0.05$ ANOVA

A.

Dose	Artesunate		Dihydroartemisinin	
	weight (g)	circumference (inches)	weight (g)	circumference (inches)
Control	2.0±0.13	4.05±0.2	2.0±0.13	4.05±0.2
½ Clinical dose	2.1±0.09	3.0±0.21*	2.05±0.08	3.8±0.14*
Clinical dose	2.15±0.1	4.0±0.12	2.2±0.51	4.0+0.03
2 x Clinical dose	2.1±0.5	3.75±1.0	2.0±0.18	3.9+0.2*

Dose	Artesunate		Dihydroartemisinin			
B.	weight (g)	circumference (inches)	weight (g)	circumference (inches)	weight (g)	circumference (inches)
Control	2.0±0.13	4.05±0.2	2.0±0.13	4.05±0.2	2.0±0.13	4.05±0.2
½ Clinical dose	1.95±0.4	3.9±0.11	1.9±0.58	3.5±0.1	1.25±0.08	3.7±0.4*
Clinical dose	1.85±0.1	4.2±0.3	1.85±0.1	4.0±0.1	2.25±0.1	4.15±0.6
2 x Clinical dose	1.4±0.1	3.15±0.2*	2.0±0.3	3.4±0.05*	1.65±0.05	4.1±0.14

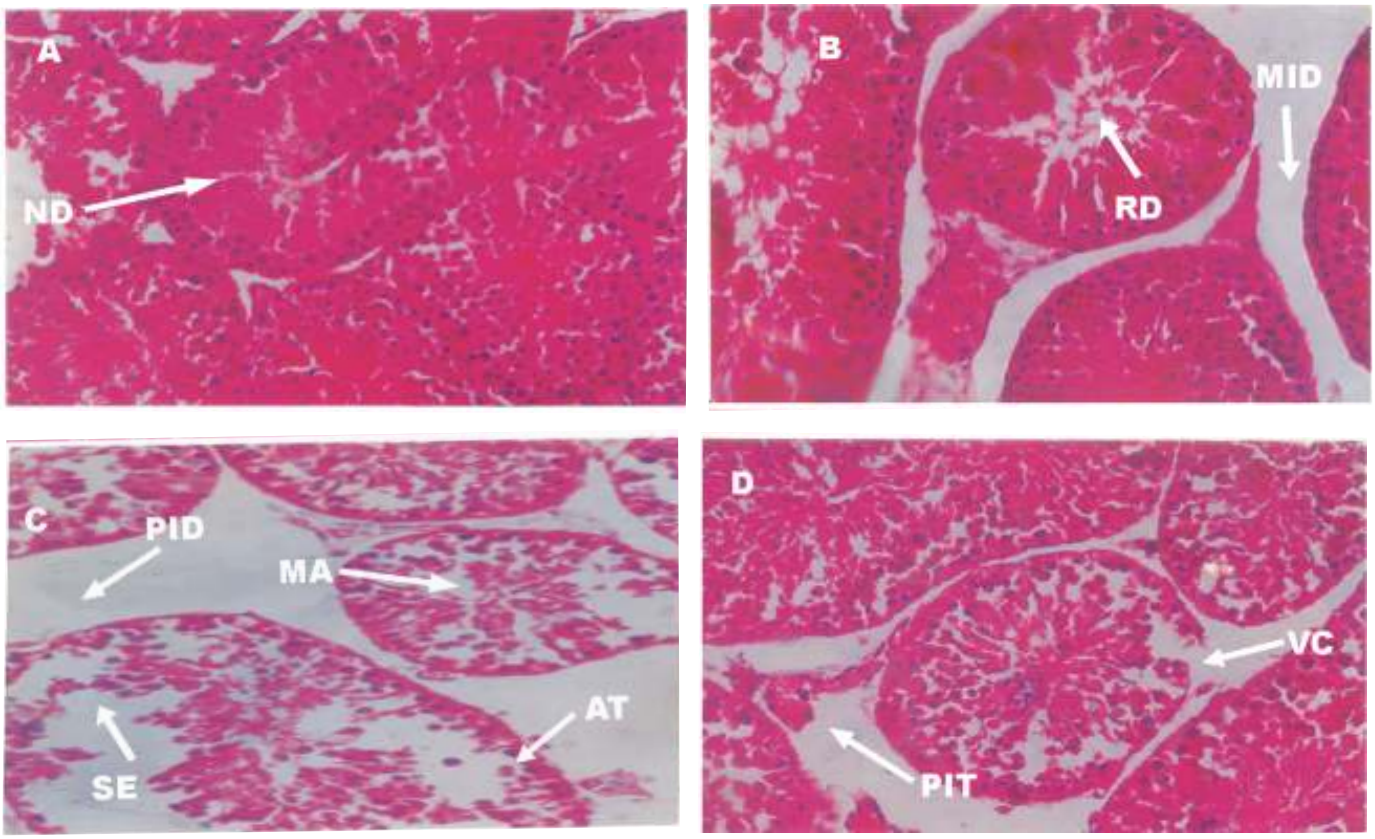

FIGURE 1: Photomicrographs showing the effects of different doses of dihydroartemisinin (Alaxin) on the testicular histology of the male guinea-pig (× 400). A- *Control*, showing normal architecture of testis with normal cell differentiation (ND); B- *half clinical dose (0.55mg/kg)*, showing moderate degree of interstitial edema (MID) and reduced sperm production (RS). C- *clinical dose (1.1 mg/kg)*, showing organ degeneration with pronounced vauolation/swelling of seminiferous epithelium (SE), pronounced interstitial edema (PID), atrophy and poor cell differentiation of sperm cells (AT) and maturation rest (MA); and D- *double clinical dose (2.2 mg/kg)*, showing pronounced interstitial edema (PIT) and vacuolation of the seminiferous tubular cells with damage to the basement membranes (VC).

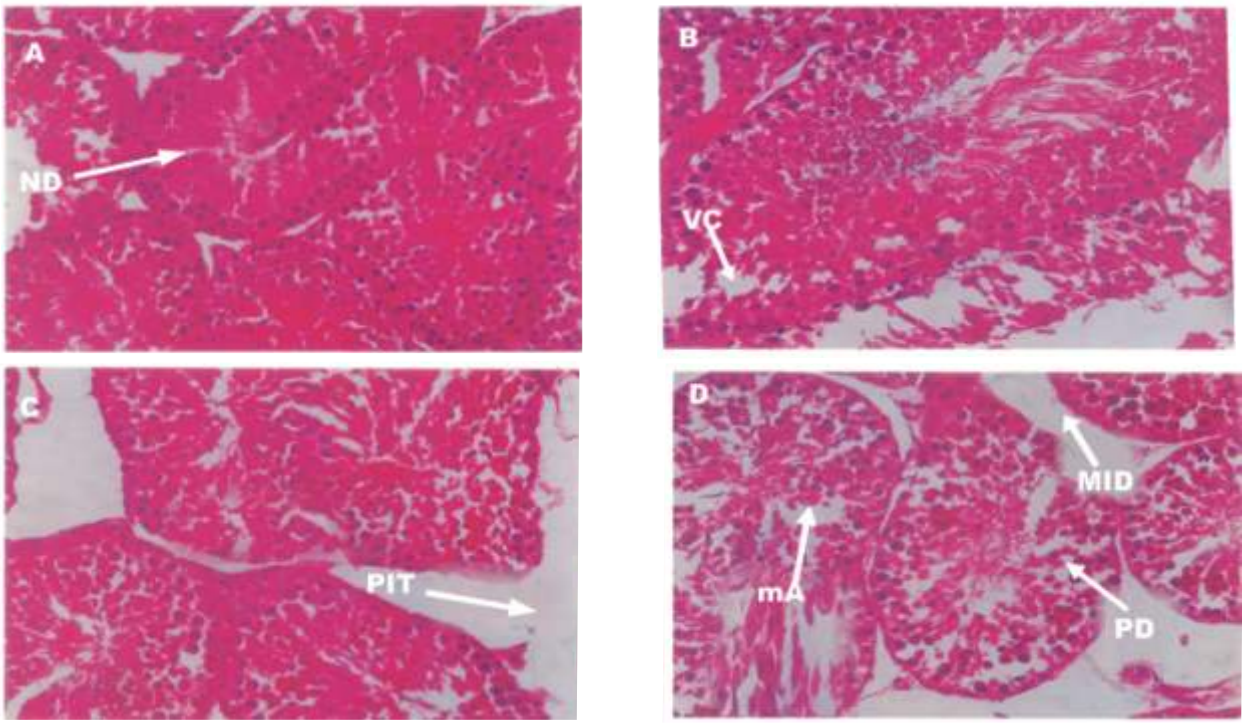

FIGURE 2: Photomicrographs showing the effects of different doses of artesunate (Arinate) on the testicular histology of the male guinea-pig (× 400). A- *Control*, showing normal architecture of testis with normal cell differentiation (ND); B- *half clinical dose (0.55 mg/kg)*, showing vacuolation and inflammation of cells of the seminiferous tubules (VC); C- *clinical dose (1.1 mg/kg)*, showing pronounced edema (PIT) and D- *double clinical dose (2.2 mg/kg)*, showing marked interstitial edema (MID), poor differentiation of sperm cells (PD) and moderate maturation arrest (mA).

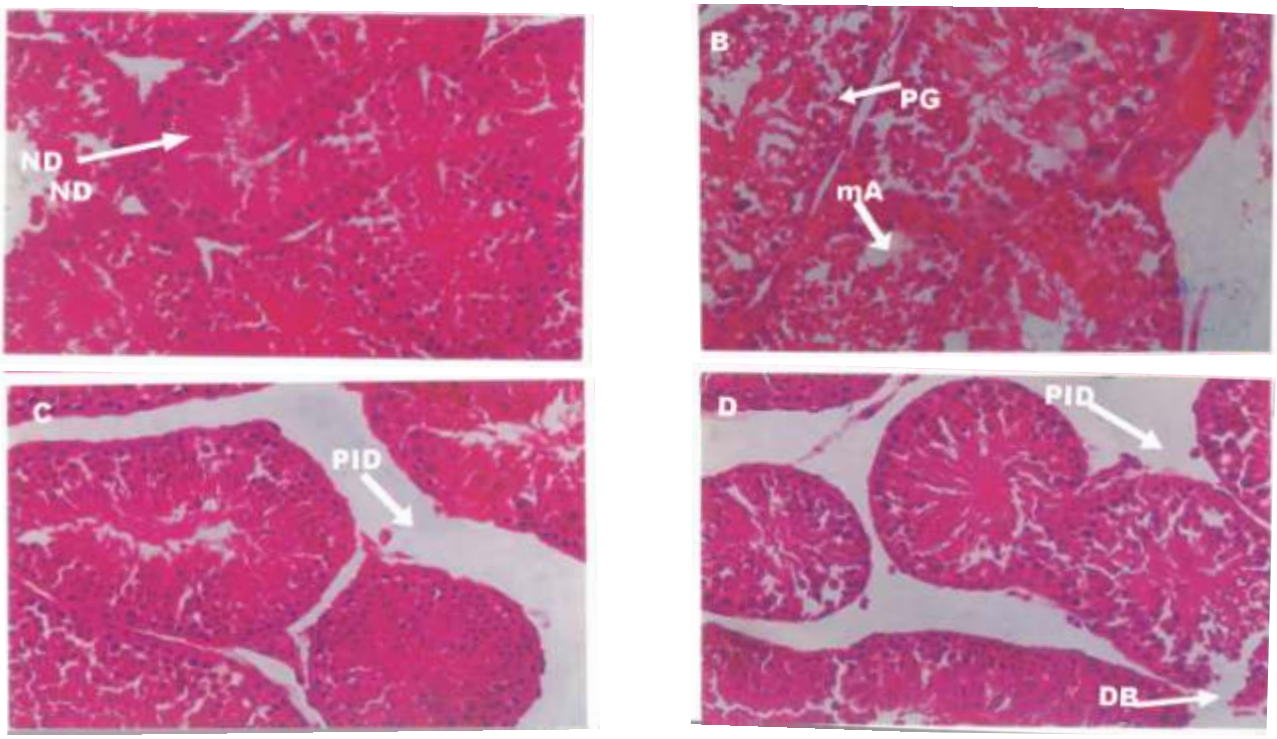

FIGURE 3: Photomicrographs showing the effects of different doses of artesunate/ sulfadadoxine/pyrimethamine (Farenax) on the testicular histology of the male guinea-pig (× 400). A- *Control*, showing normal histology of testis with normal cell differentiation; B- *half clinical dose (0.55 mg/kg)*, showing poor development of germ cells (PG) and mild maturation arrest (mA); C- *clinical dose (1.1 mg/kg)*, showing inflamed testis characterized by pronounced interstitial edema (PID); and D- *double clinical dose (2.2 mg/kg)*, showing pronounced interstitial edema (PID) and destruction of basement membrane of seminiferous epithelium

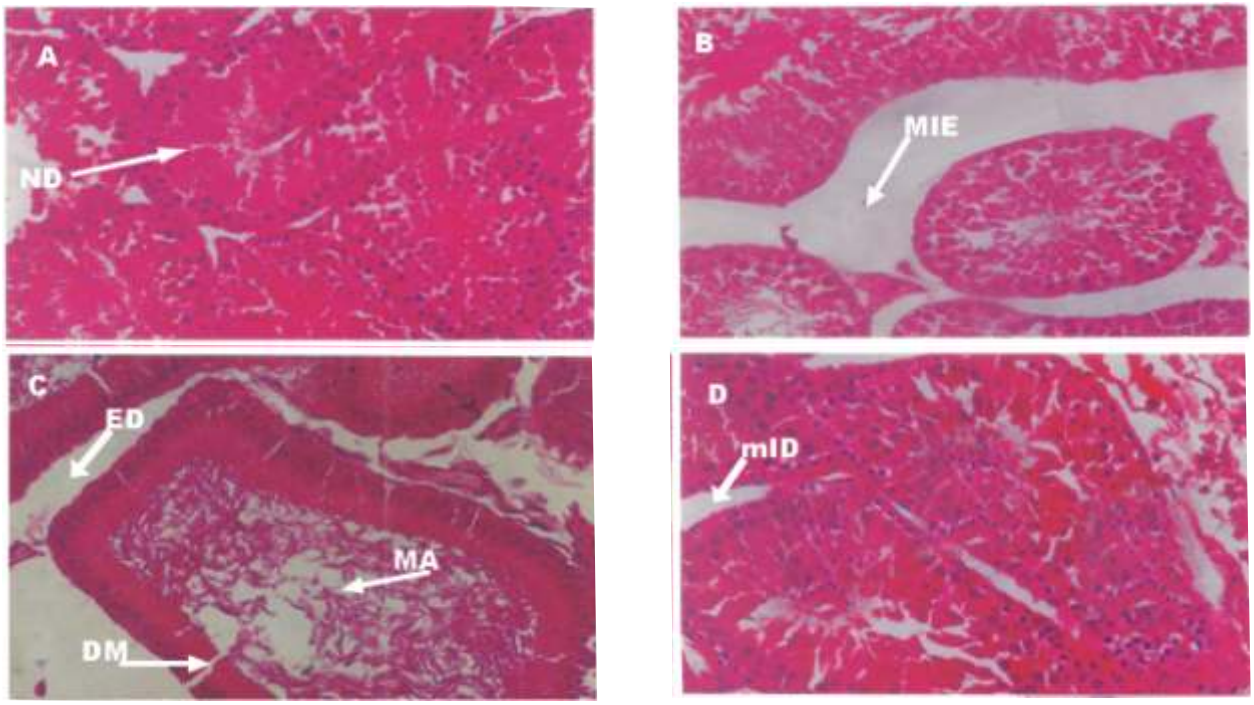

FIGURE 4: Photomicrographs showing the effects of different doses of artesunate/ amodiaquine (Dart) on the testicular histology of the male guinea-pig (× 400)**.** A- *Control*, showing normal architecture and cell differentiatiion (ND); B- *half clinical dose (0.55 mg/kg)*, showing marked interstitial edema (MIE); C- *clinical dose (1.1 mg/kg)*, showing swelling of the interstitial cells (ED), poor differentiation of germ cells and damage to the basement membrane of the seminiferous tubules (DB), depressed spermatogenesis and maturation arrest (MA); and D- *double clinical dose (2.2 mg/kg)*, showing normal histology of testis with mild inflammation of interstitial cells (mID).

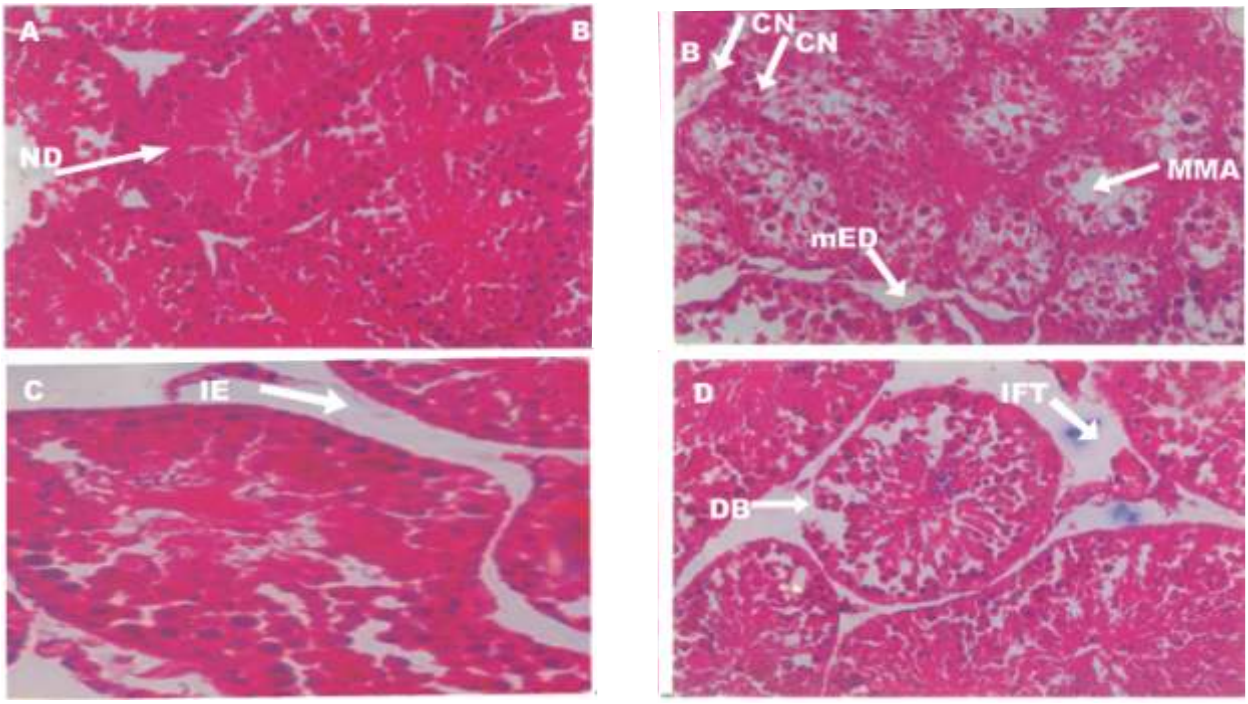

FIGURE 5: Photomicrographs showing the effects of different doses of artemether/ lumefantrine (Coartem) on the testicular histology of the male guinea-pig (× 400). A- *Control*, showing normal morphology of testis with normal differentiation of sperm cells (ND); B- *clinical dose (0.55 mg/kg)*, showing mild edema (mED), marked maturation arrest with interruption of sperm production (MMA), eosinophilia of cytoplasms of spermatocytes, shrinking of nuclei and early signs of cell necrosis (CN); and C- *clinical dose (1.1 mg/kg)*, showing minor histopathological alterations (IT); and D- *double clinical dose (2.2 mg/kg)*, showing inflammation of the interstitial cells (IE); and destruction of the basement membrane of seminiferous epithelium (DB).

DISCUSSION

In this study, the histopathological effects of artemisinin derivatives (artesunate and dihydroartemisinin) and some artemisinin-based combination therapies or ACTs (artesunate/amodiaquine, artesunate /sulfadoxine/ pyrimethamine and artemether/ lumefantrine) on the testis of male guinea-pigs were investigated.

Artemisinin or 'Quighaosu' is obtained from the decoctions of the leaves of the Chinese medicinal plant known as *Artemisia annua*, which possess potent anti-plasmodial activity (Meshnick et al 1996). Artesunate is a semi-synthetic derivative of artermisinin, while dihydroartemisinin is the active metabolite of artemisinin and its derivatives. Both agents have high efficacy against all *Plasmodium* species and are widely used as choice drugs in the treatment of malaria (Price et al 1996, White 1997). However, studies had shown that artemisinin-based combination therapies (ACTs) are better antimalarial agents than the single artemisinin derivatives with higher cure rates (White 1997, Haynes 2001). These ACTs include artesunate/amodiaquine, artesunate/ sulfadoxine/ pyrimethamine, artemether/ lumefantrine and artesunate/mefloquine. The ACTs have also been preferred to the partner agents because of resistance and toxicity (White 1997, Haynes 2001, Nosten and White 2007).

The partner agents (e.g. amodiaquine and Sulfadoxine/pyrimethamine) are relatively susceptible to most *Plasmodium* species (Wongsrichanalai et al 2002, White 2004). Amodiaquine is a 4-aminoquinoline, been used in the treatment of uncomplicated chloroquine-resistant plasmodium falciparium infections (Heppner and Ballou 1998). Sulfadoxine/ pyrimethamine is a fixed combination of a long-acting sulfonamide and the antifolate pyrimethamine. These are synergistic against sensitive parasites including Plasmodium species. Toxicities and resistance of amodiaquine and sulfladoxine / pyrimethamine to the malaria parasite have limited the use of these drugs and rendered them less effective in the treatment of malaria and makes the ACTs preferable agents (Nwanyanwu et al 1996, Koella 1998, Wongsrichanalai et al 2002, White 2004). However, many antimalarial agents including artemisinins had been reported to be gonadotoxic and antiandrogenic in male laboratory animals (Okalanwo and Ashiru 1998, Raji et al 2005, Nwanjo et al 2007).

In our earlier studies with some of these antimalarial agents, they caused significant ($p<0.05$) decreases or inhibitions in serum testosterone level, total sperm count and sperm motility, with increases in the number of abnormal sperm cells (morphology), debris and premature/primordial cells (Obianime and Aprioku 2009a). The effects were maximal at the subclinical doses and synergistic in the ACTs, compared to those of the individual partner agents (Obianime and Aprioku 2009a). In this study, artesunate, dihydroartemisinin, artesunate/amodiaquine, artesunate/ sulfadoxine/pyrimethamine and artemether/ lumefanatrine caused a dose-dependent distortion of the testicular architecture, causing significant ($p<0.05$) decrease in testicular circumference, little effects on testicular weight and inflammation of testicular cells. These responses were generally characterized by edema, eosinophilia, different degrees of maturation arrest, thus showing a positive correlation with the spermatic dysfunction observed in our earlier study. This is inconsistent with the earlier report of Nosten and White (2007) and novel as this is the first report of its kind.

The male germinal cells are particularly sensitive to oxidative stress, which easily compromises their functions (Illingworth et al 1996, Sharma and Agarwal 1996). In our earlier work, these agents also caused an increase in the basal concentrations of prostatic acid phosphatase, creatinine and urea, which are indicative of oxidative effects (Obianime and Aprioku 2009b). Increases in the serum phosphatase, creatinine and urea levels are indices for general toxicity (Perrone et al 1992,

Gaspari et al 1998). However, an increase in prostatic acid phosphatase level is a specific index of testicular toxicity (Yam 1974, Lin et al 1980), showing that these agents may cause direct toxicity in the prostate/testicular regions. It is also known that artemisinins and some antimalarial agents exert their antimalarial actions through generation of reactive oxygen species (Jefford 2001) by stimulating PKC and PKC isoforms (Kim et al 2003). This may account for the testicular toxicity obtained in this study. Increase PKC stimulation, which is associated with an increase in the production of reactive oxygen species (ROS) especially superoxide anion, hydrogen peroxide and hydroxyl radicals, can result in lipid peroxidation, membrane protein and DNA damage, which can cell proliferation and carcinogenesis (Bagchi et al 1996, Shohda et al 2001). Furthermore, ROS are capable of causing cell apoptosis, necrosis and destruction (Habeebu et al 2000).

In conclusion, artemisinin may be injurious to the testicular cells/tissues in the guinea-pig. The result of this study is very important in view of the dependence and frequent use of ACTs in the treatment of malaria. Secondly, because of the double problems of prevalence of malaria and self medication in most tropical regions including Nigeria, there is a high tendency of using the subclinical, clinical and high doses of these drugs.

The results obtained in this study may be due to oxidative trauma induced on the testicular tissues, especially the Leydig and Sertoli cells by the drugs. This may also affect plasma testosterone concentration and function, resulting in impaired spermatogenesis and testicular organ degeneration.

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