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Experimental Research Article

Effects of long-term repeated treatment with artemisinin-based combination therapy on the reproductive potential of male mice

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ABSTRACT

Introduction: In endemic malarial nations, repeated use of antimalarial drugs has increased due to resistance, misuse, and unrestricted availability, which could contribute to infertility rates. Therefore, we investigated the effects of long-term repeated treatment with two commonly prescribed artemisinin-based combination therapies, artemether/lumefantrine (A/L) and artesunate-amodiaquine (A/A), on reproductive potential in mice.

Methods: Sixty male mice were divided into three groups: control, A/L, and A/A treatment. Mice underwent treatment for three consecutive days per week, and this regimen was repeated every two weeks for a total of six cycles. Sperm parameters were evaluated after the 1st, 2nd, 3rd, and 6th exposure cycles, after which treated male mice were paired with female mice for mating.

Results: Sperm viability was significantly reduced by 21% (P<0.001) following the 6th exposure to A/L, whereas the 2nd, 3rd, and 6th exposures to A/A resulted in significant decreases in sperm viability of 26% (P<0.001), 12% (P<0.01), and 31% (P<0.001), respectively, compared to the control group. Treatment with A/A during the 3rd and 6th periods led to a significant decline (P<0.001) in sperm mass activity by 20% and 28%, respectively, compared to the control group. However, long-term therapeutic exposure to A/L or A/A did not affect testosterone levels, epididymal content, or the ability to impregnate female mice.

Conclusion: Long-term treatment with A/L or A/A did not affect testosterone levels or epididymal content. However, a decrease in sperm viability was observed, even though the mice remained fertile.

Keywords:
Infertility
Artemisinin
Sperm cells
Testosterone

Introduction

Artemisinin-based combination therapies (ACTs) have revolutionized the treatment of malaria, which is a significant global health burden. ACTs, such as artemether/lumefantrine (A/L) and artesunate/amodiaquine (A/A) have proven highly effective in reducing the morbidity

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and mortality associated with malaria infections(Audu et al., 2023b; Hanboonkunupakarn and White 2016; Koko et al., 2022; Nsanzabana 2019; Pousibet-Puerto et al., 2016; Shibeshi et al., 2021; W.H.O 2021). However, the rise in malaria cases in endemic countries, the development of antimalarial resistance, prophylaxis, misuse, and unrestricted availability of these drugs have led to increased rates of antimalarial drug exposure(Ajayi and Ukwaja 2013; Ajogbasile et al., 2022; Anyanwu et al., 2017; Nwokolo et al., 2018; Omole 2010). The increased use of antimalarial drugs has raised concerns regarding their potential adverse effects on various physiological systems, including reproductive functions (Abolaji et al., 2012; Aprioku 2018; Audu et al., 2023a; Samuel et al., 2018).

Infertility affects a significant portion of the global population, with approximately one in six people experiencing it at some point in their lives (W.H.O 2023). Interestingly, the percentage of infertility attributable to males varies widely, ranging between 20-70% in different nations. (Agarwal et al., 2015). Drug exposure plays a significant role in male infertility (Durairajanayagam 2018; Jafari et al., 2021; Kumar and Singh 2015; Uadia 2015). Long-term exposure to drugs can have detrimental effects on male fertility by increasing levels of drug metabolites in the body. These metabolites can directly affect sperm cells by entering the seminal fluid (Alghobary and Mostafa 2022; Mann et al., 2020; Patricio et al., 2016; Sansone et al., 2018), causing various alterations in sperm physiology, metabolism, and genetic composition, ultimately, this could potentially contribute to infertility in the affected individuals (Patricio et al., 2016; Tavilani et al., 2005). Although the precise mechanism by which ACTs lead to reproductive toxicity is not yet comprehensively understood, experts have suggested that reactive oxygen species production during artemisinin metabolism may cause oxidative stress in the testicular surroundings. This could eventually culminate in DNA damage and compromise germ cell capabilities (Asadi et al., 2017; Audu et al., 2023a; Bui et al., 2018; Daikwo 2018; Kavishe et al., 2017; Ko et al., 2014; Leisegang and Dutta 2021; Villaverde et al., 2019; Wagner et al., 2018).

Previous studies have explored the acute, short-, and long-term effects of artemisinin and its derivatives on sperm quality, fertility, and testicular histology in various animal models (Aprioku and Mankwe 2018; Daikwo 2018; Mofio et al., 2020; Samuel et al., 2018). Interestingly, none of the existing studies have specifically investigated the effects of long-term, weekly repeated treatment with ACTs on male mouse reproductive parameters, and most studies on long-term treatment have used ACTs as a single continuous treatment for up to 14 days (Aprioku 2018; Aprioku and Mankwe 2018; Daikwo 2018), and 21 days(Mofio et al., 2020), not as the three days weekly repeated therapy, which reflects clinical reality. Furthermore, there is a lack of comparative research examining the effects of single treatments versus long-term repeated interval treatments, spanning three or six consecutive repeated instances. This comparison is particularly important as it provides valuable insights into the potential consequences of repeated therapeutic ACTs use as a prophylactic measure or misuse on male reproductive potential. To address these knowledge gaps, this study employed a rigorous experimental design using male mice as a model system to evaluate the long-term effects of repeated treatments with two commonly prescribed ACTs, A/L and A/A, on male reproductive potential.

Material and methods

Acquisition and care of animals

Sixty male and 48 female mature BALB/c mice were used in this study. These mice had an average weight of 25 g and were eight weeks old. The Institute of Advanced Medical Research and Training at the University College Hospital (UCH) in Ibadan, Nigeria. They were provided with standard feed manufactured by Ladokun Feed Limited, located in Ibadan, Oyo State, Nigeria. To ensure proper care, the mice were housed in plastic cages with dry wood shavings that served as bedding materials. They were provided with standard feed. Throughout the study, mice had unrestricted access to both food and water, ensuring that their nutritional needs were always met. Additionally, they were subjected to a consistent light-dark cycle of 12 h of light, followed by 12 h of darkness.

Dosage and administration of antimalarial drug

Lumartem (A/L) Antimalarial Tablet (20 mg/120 mg), was purchased from Cipla Pharmaceuticals Limited (Mumbai, India) while Camosunate (A/A) (100 mg/300 mg) was obtained from Geneith. Pharmaceuticals Limited in Lagos, Nigeria.This dose was administered at six

FIGURE 1. Diagram illustrating the study design.

intervals, 8, 24, 36, 48, and 60 h. Simultaneously, the A/A group received A/A at doses of 2.86/8.58 mg/kg/d three consecutive days per week. These dosages have been established in previous studies by (Daikwo 2018; Otuechere et al., 2012). The clinical doses of both drugs were administered orally using an intragastric feeding needle to ensure accurate delivery of the medication.

Research design

Sixty males were randomly assigned to three groups using computer-generated numbers, as described by (Johnson and Besselsen 2002). Each group consisted of 20 male mice housed in four cages, each containing five mice. The sample size was determined using the formula defined by(Charan and Kantharia 2013).

Group 1: Administered only distilled water,

Group 2: Administered therapeutic doses of A/L

Group 3: Administered therapeutic doses of A/A.

The administration was carried out three days per week (mimicking the standard treatment regimen for A/L and A/A), and the treatment was repeated every two weeks, for six cycles of exposure (Fig. 1). After the 1st, 2nd, 3rd, and 6th exposure periods, three mice blood and epididymis were obtained to test for testosterone and sperm parameters (Fig. 1). Two male mice from each group were grouped with two female mice for mating after the 1st, 2nd, 3rd, and 6th treatment period, and the number of successful pregnancies was recorded (Fig. 1). The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 85-23, updated in 1996).

Epididymal sperm parameters

The caudal epididymis was individually placed in a petri dish and minced in normal saline (1 mL) to create a sperm suspension. Triplicates (10 µL) were collected from each suspension and placed on microscope slides for analysis. At 40× magnification, both motile and non-motile spermatozoa were counted in ten randomly selected fields to assess sperm mass activity. The percentage of motile spermatozoa was calculated based on

the average motility percentage across all the counted fields.

To determine sperm viability, eosin, and nigrosin were mixed with the spermatozoa. A smear of the sperm-eosin-nigrosin mixture was air-dried and examined using a 40× objective lens. The proportion of live and dead sperm cells was counted to calculate percentage viability. Live sperm cells remained unstained, whereas dead sperm cells absorbed stain. The sperm concentration was measured using an improved Neubauer counting chamber. The chamber was prepared, filled with diluted seminal fluid (1:20), and allowed to stand in a moist chamber for 15 minutes, morphologically mature sperm cells were counted using at 40× magnification. Sperm smears were stained on microscope slides to assess the sperm morphology and slides were examined under bright-field optics at 100× magnification with oil immersion. Approximately 200 spermatozoa were counted, the percentage of abnormal spermatozoa was calculated, and types of abnormalities were noted (Aprioku 2018; Aprioku and Mankwe 2018; Baker 2007; W.H.O 2010).

Testosterone Assays

Blood samples were obtained from each mouse through cardiac puncture, and serum was used to determine testosterone levels. This was performed using an enzyme-linked immunosorbent assay (ELISA) method (Raji et al., 2005). The ELISA kits were manufactured by Inteco Diagnostics London UK (Bio-Inteco) (LOT number: B-I-118101602), and the assay was performed according to the manufacturer's instructions.

Histopathology of the epididymis

Epididymis from mice in each group were fixed in Bouin's fluid and processed for paraffin embedding. Sections of 4µm thickness were taken serially with a rotary microtome, processed in an alcohol-xylene series, and stained with hematoxylin and eosin (H&E). The prepared slides were examined at × 400 magnification.

Statistical analysis

Raw laboratory data were analyzed, and graphs were generated using the GraphPad Prism 9.0 application. One-way analysis of variance (ANOVA) was used to determine the significance of the differences between the treated and control groups. The results are shown graphically as the mean standard error of the mean (SEM) (n =3). The Shapiro-Wilk test was initially employed to check whether the distribution of the data was normal. The significance of the mean values *, **, and *** indicate significant differences at $P< 0.05$, $P< 0.01$, and $P<0.001$, respectively, compared with the relevant control groups.

Results

Effects of repeated treatment with A/L or A/A on mice testosterone, Sperm mass activity, viability, and concentration

Testosterone levels in the experimental groups exposed to A/L and A/A after the 1st, 2nd, 3rd, and 6th treatments did not demonstrate statistically significant alterations compared to those in the control groups (Fig. 2A).

No statistically significant changes were observed in sperm mass activities following the 1st and 2nd treatments with A/L and A/A when compared to the control group (Fig. 2B). Significant reductions in sperm mass activity were observed following the 3rd and 6th exposure periods to A/A, with decreases of 20 % and 28 % ($P<0.001$), respectively, compared to the control group (Fig. 2B). Treatment with A/L resulted in an 8 % increase in sperm activity after the 3rd treatment compared with that in the control group (Fig. 2B).

No statistically significant changes were observed in sperm viability following the 1st treatment with either A/L or A/A compared with the control group (Fig. 2C). Treatment with A/L resulted in a significant 16 % increase in sperm viability after the 3rd exposure period compared with the control group (Fig. 2C). A significant reduction in sperm viability was observed after the 2nd, 3rd, and 6th exposures to A/A, with decreases of 26 % ($P<0.001$), 12 % ($P<0.01$), and 31 % ($P<0.001$), respectively, compared with the control group (Fig. 2C). However, treatment with A/L for the 6th time resulted in a significant ($P<0.001$) reduction in sperm viability by 21 % compared to the control group (Fig. 2C).

Sperm concentration was not significantly altered following treatment with A/L and A/A during the 1st, 2nd, and 3rd treatments when compared to the control group (Fig. 2D). The sperm concentration significantly increased by 117 % in the group of mice treated with A/L after the 6th exposure period compared with that in the control group (Fig. 2D).

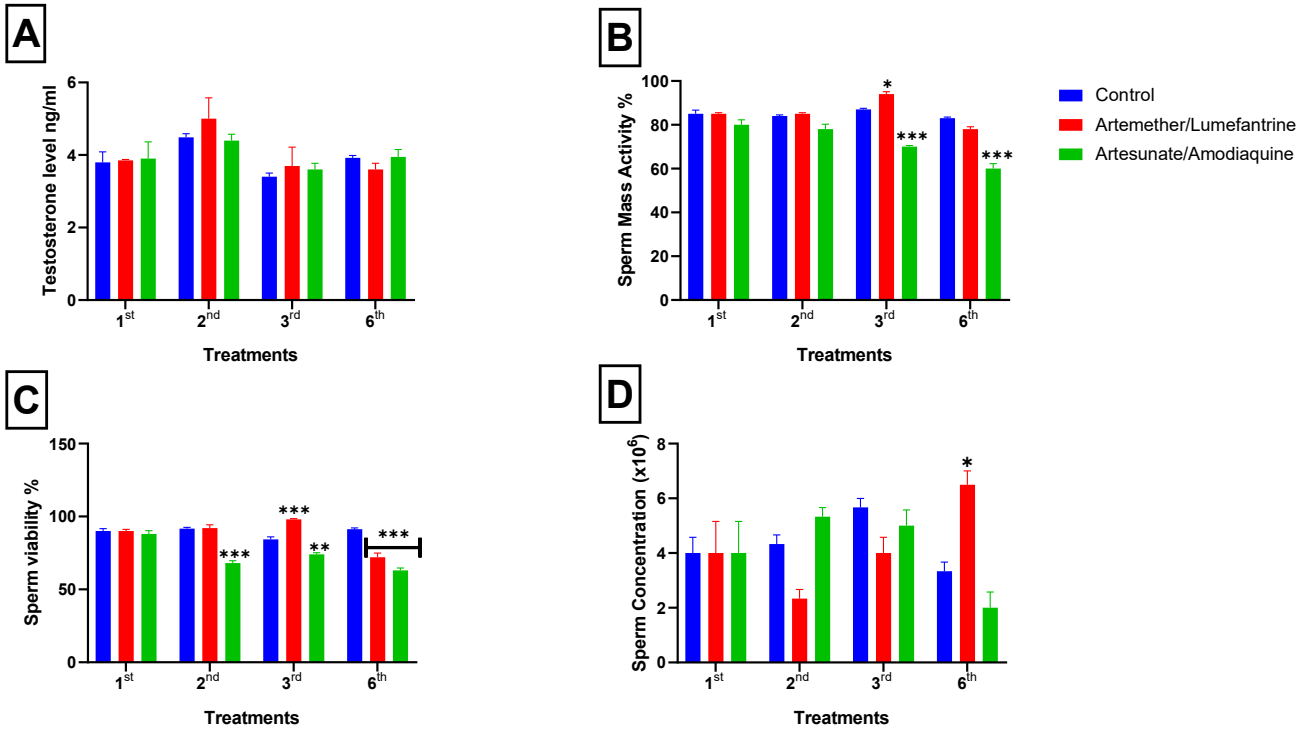

FIGURE 2. (A) Testosterone level (B) Sperm mass activity (C) Sperm viability (D) Sperm concentration after the 1st, 2nd, 3rd, and 6th exposure of mice to artemether/lumefantrine (A/L) or ar-tesunate/amodiaquine (A/A) therapeutic regime. Values are expressed as mean ± SEM (n = 3), and a, b, and c indicate significant differences at $P< 0.05$, $P< 0.01$, and $P< 0.001$, respectively, compared to the corresponding control groups.

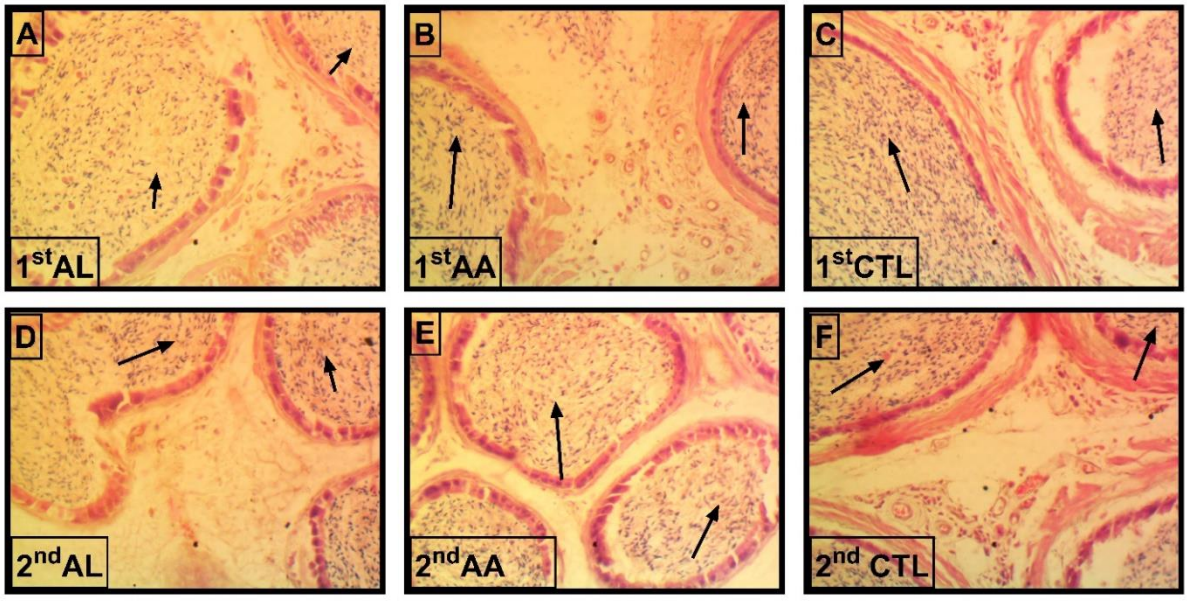

FIGURE 3. Epididymis filled with spermatozoa (arrow) (400; H & E) in mice (A) After the 1st treated with A/L; (B) After the 1st treatment with A/A(C) 1st control; after the 1st treatment with distilled water, (D) After the 2nd treatment with A/L, (E) After the 2nd treatment with A/A, and (F) 2nd Control; after the 2nd treatment with distilled water.

Effects of repeated A/L or A/A treatment on mice Sperm Morphology and epididymal content

Normal sperm cells decreased significantly (P<0.001) after the 1st treatment with A/L compared with the control group, but this effect diminished in subsequent treatments. The significantly altered sperm morphologies af-

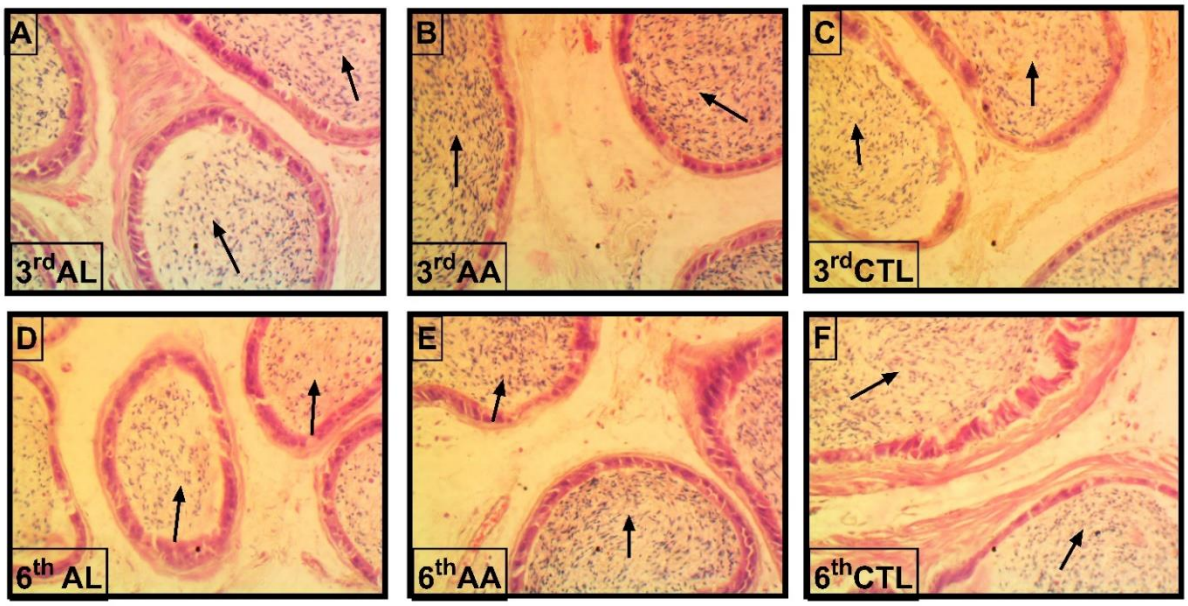

FIGURE 4. Epididymis filled with spermatozoa (arrow) (400X; H & E) in mice (A) After the 3rd treatment with A/L; (B) After the 3rd treatment with A/A(C) 3rd control; after the 3rd treatment with distilled water, (D) After the 6th treatment with A/L, (E) After the 6th treatment with A/A, and (F) 6th Control; after the 6th treatment with distilled water.

ter the 1st treatment with A/L included free head (FH), dwarf Tail (DWT), and acrosomal defect (ACD), with FH being the most significantly abnormal (P<0.001). Despite these changes, the success rate in impregnating female mice was 100% (Table 1). After the 2nd treatment with A/A, normal sperm cells decreased significantly (P<0.001) by 10 % compared with the control group, although subsequent treatments did not show significant alterations. The significantly altered sperm morphologies observed after the 2nd treatment with A/A included Bent Mid Peace (BMP), FH, and coiled tail (CT), with FH being the most significant (P<0.001). The success rate in impregnating female mice remained 100% (Table 1). Treatment with A/L and A/A after the 3rd and 6th treatment periods did not significantly alter normal sperm compared to the control group, and the success rate in impregnating female mice remained 100% (Table 1).

Histological examination of the epididymis sections in both the A/L, A/A, and control groups after the 1st, 2nd, 3rd, and 6th treatment revealed a pronounced presence of spermatozoa, densely filling the epididymal tubules (Fig. 3 and 4).

Discussion

The present study investigated the effects of long-term repeated treatment with ACTs on the reproductive potential of male mice. These results provide valuable insights into the potential effects of this administration pattern on various reproductive parameters, including testosterone levels, epididymal content, sperm mass activity, viability, and morphology.

One of the key findings of our study was that single and repeated administrations of A/L and A/A did not significantly affect testosterone levels in mice. Testosterone, the primary male sex hormone, plays a crucial role in the regulation of reproductive functions, including spermatogenesis (Nassar and Leslie 2023). The absence of a significant alteration in testosterone levels suggests that testosterone production was not significantly affected by repeated administration of artemisinin-based combination therapies. A study conducted by (Samuel et al., 2018) also revealed that therapeutic administration of A/L and A/A for three or six days did not significantly affect testosterone levels, while (Alagbonsi et al., 2021) found no alterations in testosterone levels in male rats treated with low-dose artesunate. Also, a study conducted by (Olufemi et al., 2009) demonstrated that administering A/L for short-term periods of three and six days did not have any detrimental effects on testosterone levels in rats, and (Daikwo 2015) found that prolonged administration of A/L did not significantly affect testicular testosterone concentrations in rats. However, these

TABLE 1: Mice treated with either A/L or A/A after 1st, 2nd, 3rd, and 6th period Sperm Morphology analysis and success rate in impregnating female mice

Therapeutic Treatment Exposure Period	Treatment	Normal cells (%)	Abnormal Cells (%)						Success Rate in impregnating female mice (%)
			Bent Mid Peace (BMP)	Free Head (FH)	Free Tail (FT)	Coiled Tail (CT)	Dwarf Tail (DWT)	Acrosomal Defect (ACD)	
1st	CTL	91.00±1.16	0.00±0.00	3.00±0.00	3.67±0.88	1.33±0.33	0.00±0.00	1.00±0.00	100
	AL	82.67±0.67***	0.00 ±.0.00	6.33 ±0.33***	5.33±0.33	1.00±0.00	2.67±0.33**	2.00±0.00*	100
	AA	90.33±0.88	1.00 ±0.58	4.00 ±0.58	3.00±0.58	1.00±0.00	0.67±0.67	0.00±0.00*	100
2nd	CTL	91.33±1.20	0.00±0.00	2.33±0.33	4.33±0.88	1.00±0.00	0.00±0.00	1.00±0.00	100
	AL	86.33±0.88	0.00 ±0.00	6.00±0.58***	5.00±0.58	2.00±0.00	0.67±0.67	0.00±0.00*	100
	AA	83.00±1.53***	2.00 ±0.00**	6.00±0.00***	3.00±1.52	5.00±0.58***	0.00±0.00	1.00±0.58	100
3rd	CTL	91.67±0.88	0.00±0.00	3.00±0.00	2.33±0.88	2.00±0.00	0.00±0.00	1.00±0.00	100
	AL	90.33±0.88	2.00 ±0.58**	3.33±0.33	3.67±0.67	0.00±0.00	0.67±0.67	0.00±0.00*	100
	AA	91.00±2.60	2.00 ±0.58**	4.00±0.58	2.00±1.16	0.00±0.00	0.33±0.33	0.00±0.00*	100
6th	CTL	89.00±1.16	0.00±0.00	3.33±0.67	4.67±0.33	2.00±0.58	0.00±0.00	1.00±0.00	100
	AL	93.00±1.15	0.00±0.00	0.00±0.00***	6.00±0.58	1.00±0.58	0.00±0.00	0.00±0.00*	100
	AA	91.67±1.15	2.00±0.00**	0.00±0.00***	0.33±0.33**	3.00±1.15	2.00±0.00	1.00±0.58	100

AL: Artemether/lumefantrine; AA: Artesunate/Amodiaquine; CTL: Control; Values are expressed as mean ± SEM (n = 3). *, **, and *** indicate significant differences at P<0.05, *P*< 0.01, and *P*< 0.001, respectively compared to its corresponding control group

findings are in contrast to those of a more recent study by (Okwakpam et al., 2023), which revealed that testosterone levels in rats significantly decreased following a single standard treatment with A/L.

Histological examination of the epididymis revealed abundant spermatozoa in mice treated with A/L and A/A. The epididymis serves as a storage site for mature sperm and plays a critical role in sperm maturation and motility (James et al., 2020; Marchiani et al., 2017). The presence of a high number of spermatozoa indicates that the process of sperm production and maturation was not affected by repeated administration of ACTs. This finding is contrary to those of previous studies (Mofio et al., 2020) which revealed a severe reduction in epididymal content after A/L treatment through drinking water for 21 days.

The lack of change in testosterone levels and non-reduced epididymal content observed during repeated treatments with A/L and A/A could be attributed to several factors. One possible explanation is the treatment gap between each cycle, which may have been sufficiently long to prevent any significant effects on testosterone levels. It is plausible that these drugs were efficiently metabolized and eliminated from the mouse body, thereby minimizing their effects on the hormone levels. Moreover, the absence of alterations in testosterone levels could be due to the differential sensitivity or response of mice compared with other species or humans. This notion is supported by studies conducted in pigs, which showed a decrease in testosterone levels following the administration of A/A(Aprioku 2018; Aprioku and Mankwe 2018; Obianime and Aprioku 2009), and after the A/L treatment (Aprioku and Mankwe 2018). These findings suggest that mice may have a different hormonal response to antimalarial drugs than pigs.

The study conducted by(Daikwo 2015) concluded that the prolonged administration of A/L did not have a substantial impact on sperm concentration, motility, and cell morphology in rats, while a different study conducted by (Olufemi et al., 2009) showed that short-term administration of A/L for 3 and 6 days had no detrimental effect on sperm count, motility, viability, and morphology in rats. However, our study demonstrated a decline in sperm mass activity after the 3rd and 6th exposures to A/A, and a decrease in sperm viability after the 2nd, 3rd, and 6th exposures with the 6th exposure to A/L significantly reduced sperm viability. Sperm mass activity, and viability are crucial determinants of sperm function and fertility. The observed decrease in these parameters suggests that repeated ACT administration may have detrimental effects on sperm motility and viability. These findings are consistent with those of previous studies (Aprioku 2018; Aprioku and Mankwe 2018), which reported impaired sperm function following exposure to artemisinin derivatives. The decrease in sperm mass activity and viability observed after AA exposure may be due to the amodiaquine component of this drug, which induces oxidative stress (Bisht et al., 2017; Dutta et al., 2019; Li et al., 2002; Sabeti et al., 2016). Although a single treatment with A/A and A/L did not affect sperm mass activity and viability, the changes observed in these sperm parameters after repeated exposure could be ascribed to the long-term accumulation of oxidative stress in sperm cells caused by prolonged A/A and A/L exposure. This finding suggests that extended exposure to either A/L or A/A may adversely affect the overall viability and health of sperm cells.

Contrary to our expectations, no significant effects on sperm normal cells were observed after repeated A/L or A/A treatments in the 3rd and 6th cycles. This aligns with the findings of Aprioku and Mankwe (2018). This suggests that the changes observed in sperm mass activity and viability, as mentioned earlier, do not correspond with alterations in sperm normal cells. These findings imply that the effects of repeated A/A and A/L treatments on sperm characteristics may vary, although a decline in sperm viability and the absence of significant changes in sperm normal cells indicate a certain level of resilience after repeated treatment with these drugs.

Furthermore, this study demonstrated a 100 % success rate in impregnating female mice after repeated A/L or A/A treatment of male mice. This suggests that despite the observed effects of repeated treatment on sperm mass activity and viability, the fertility potential of male mice remained unaffected. The implications of this result are noteworthy because they indicate that the alterations in sperm mass activity and viability observed in this study were not associated with substantial impairment in sperm morphology. The ability to successfully impregnate female mice underscores a compensatory mechanism within the reproductive system that may compensate for any temporary disruptions caused by repeated use of A/L and A/A.

Conclusion

The results of this study revealed that repeated treatments with A/L or A/A did not have any significant detrimental effects on testosterone levels, epididymal content, or sperm concentration. However, there was a decrease in sperm viability among the mice, although this did not lead to infertility. It is important to note that this study has some limitations, and further research is needed to thoroughly examine the impact of A/L and A/A on reproductive potential in male mice, particularly when these drugs are used as prophylactic measures or in the event of drug misuse. Conducting additional research will improve our understanding and enable more accurate application of these findings in human contexts.

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Conflict of interest

The authors report no competing interests.

Ethics approval

The experimental protocol was approved by the College of Veterinary Medicine Research Ethics Committee of the Federal University of Agriculture, Abeokuta (FUNAAB/COLVET/CREC/2019/07/01).

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