



SEPARATE AND COMBINED SUB-ACUTE EFFECTS OF ARTESUNATE, AMODIAQUINE ON SPATIAL MEMORY AND HIPPOCAMPAL MORPHOLOGY OF ADULT WISTAR RATS

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ABSTRACT

We evaluated the sub-acute effect of artesunate (AS), amodiaquine (AQ) and AS plus AQ on the structure and function of the hippocampus of adult Wistar rats. Forty adult male Wistar rats weighing between 110-215 g, were divided into 4 groups (n=10); group A-control (CT) rats administered distilled water, group B-4 mg/kg, AS, group C-10 mg/kg, AQ and group D- 4 mg/kg, AS + 10 mg/kg, AQ body weight. Drugs were administered orally for 3 days and neurobehavioral tests (Morris water maze) for spatial memory and cognition done from day 11 to 14. The rats were sacrificed on the day 15 and blood sample collected for full blood count. The rat brain of all groups was excised, and the hippocampus dissected out, fixed in 10% formol-saline and processed for histology and immunohistochemical (glial fibrillary acidic protein, GFAP (astrocytes) and inducible nitric oxide synthase, iNOS (oxidative stress)) studies. Data were analysed by one-way ANOVA at $p < 0.05$. Sub-acute evaluation showed that the AQ-treated rats had significantly increased swimming time and distance, red blood cell (RBC), white blood cell (WBC), haemoglobin (Hb) and packed cell volume (PCV) compared with the CT and other groups. Histologically, there was decreased Cornus Ammonis 1 (CA 1) pyramidal neurons in the hippocampus of the AS and AQ-treated rats compared with the CT group. Increased astrocyte population was observed in the hippocampus of AS and AQ groups compared with the CT and AS+AQ groups, as well as increased iNOS expressions compared with the CT group. Sub-acute evaluation of the adult rat hippocampus indicated that amodiaquine decreased spatial memory and increased blood cell counts, while artesunate and amodiaquine induced oxidative stress resulting in pyknosis of and decreased pyramidal CA1 neurons and caused astrogliosis.

Key words: Artesunate, Amodiaquine, Hippocampus, Neurobehaviour, Histology, immunohistochemistry

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INTRODUCTION

Artemisinin-based combination therapy (ACT) has been endorsed by the World Health Organization (WHO) as the "policy standard" for all malaria infections in areas where *Plasmodium falciparum* is the predominant infecting species (Takala-Harrison and Laufer, 2015; WHO, 2015). The ACTs have been documented to increase cure performance rate, decrease resistance ability of parasites, and reduce transmission of parasites that are drug resistant. The success of ACTs is to prevent transmission of infected plasmodium parasites and reduce the chance of parasite recrudescence (WHO, 2015). Artesunate-amodiaquine combination (ASAQ) is one of the

most used ACTs available. The ASAQ is prepared in diverse formulations, such as in single fixed-dose or separate co-package. The efficacy of ASAQ in the treatment of malaria in malaria endemic regions is well documented (Adjuik *et al.*, 2002; Abacassam *et al.*, 2004; Mutabingwa *et al.*, 2005 and Martin *et al.*, 2006). Although, artemisinin derivatives were reported to have little or no neurological adverse effects in humans (Gordi and Lepist, 2004), there is poor documentation of their histological activities using experimental animals (WHO, 2000; Wilmer *et al.*, 2003). Therefore, continuous scientific studies involving treatment with clinically relevant

doses of artemisinin and its derivatives would be necessary. In addition, self-medication is quite common and purchase of antimalarials in the open market is rampant among victims of malaria (Akanbi *et al.*, 2005), therefore the possibility of overdose administration and misappropriation in the usage of the drugs are very common, all of which could lead to the toxic effects of the drugs on some body organs,

including the brain (Jaeger *et al.*, 1987; Agboruche, 2009 and Izunya *et al.*, 2010). This study was designed to evaluate at sub-acute level, the separate and combined effects of artesunate and amodiaquine on the spatial and cognitive memories, haematological indices, histology, and immunohistochemistry of the hippocampus of adult Wistar rats.

METHODOLOGY

Forty adult male Wistar rats (*Rattus norvegicus*) weighing between 110 and 200 g procured from the animal house of the Department of Veterinary Physiology were used for this experiment. The experimental animals were handled and used in accordance with the guidelines provided by the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC). They were kept in standard laboratory conditions under 24-hour light condition at room temperature of 25°C. The animals were made to have access to feed and water *ad-libitum* throughout the period of the experiment.

The 40 animals were randomly divided into four (4) groups (n=10) as follows:

1. Group A - control group (CT) received distilled water
2. Group B – Received 4 mg/kg body weight artesunate (AS)
3. Group C – Received 10 mg/kg body weight amodiaquine (AQ)
4. Group D – Received 4mg/kg body weight AS + 10 mg/kg body weight AQ.

The doses used above represent the standard therapeutic doses of the AS and AQ and mimics a situation in which the ACT is used without any malaria infection (Adebayo *et al.*, 2011). Drugs were administered orally once daily between the hours of 8:00 and 10:00 am for 3 days.

Morris water maze test: it is a neurobehavioural test of spatial memory of the rats, a function of the hippocampus. This was carried out on the 11th, 12th, 13th (acquisition

trials) and 14th days (probe trial) of the experiment for sub-acute study. Briefly, the rats were placed in a pool of water where they must use visual cues to remember the location of a hidden platform just below the water surface. The water was made opaque by adding milk and was periodically drained for cleaning and disinfection. The 10 cm circular escape platform was made from water-resistant material. Animals were dried with towels. The animals found not locating the platform within 2 minutes were manually guided to the platform. Probe trials (transfer tests) were also used to assess the rat's ability to retrieve information learned in previous hidden platform tests. Total swimming time, swimming distance, time spent in each quadrant and the frequency of crossing the escape quadrant were quantified by means of hand-scoring according to the method of Morris, 1984 modified by Brandeis *et al.*, 1989, D'Hooge *et al.*, 2001 and Vorhees *et al.*, 2006. Blood sample (1 ml) was withdrawn from each group of animals by ocular puncture on day 14. This was done by puncturing the medial canthus of the animal's eye with EDTA capillary bottles. The following haematological indices were estimated: Packed cell volume (PCV), haemoglobin concentration (Hb), red blood cell (RBC) count, white blood cell (WBC) count, and the differential white blood cell count (lymphocytes, monocytes, neutrophils, basophils and eosinophils) by an autohaematological analyser machine at the haematology laboratory of the University College Hospital (UCH), Ibadan, using the method of Baker *et al.* (1998).

The rats were then sacrificed by quick cervical dislocation and the brain removed from the cranial cavity and the hippocampus dissected out, fixed in 10% neutral buffered formalin and processed using routine paraffin wax embedding technique and stained with haematoxylin and eosine for the histology of the hippocampus. Immunohistochemical techniques were also employed to evaluate;

- i. Astrocyte population using the Glial Fibrillary Acid Protein (GFAP)
- ii. Oxidative stress using Inducible nitric oxide synthase (iNOS) method as

described by Delcambre et al. (2016).

Images were captured from the hippocampus with a 500-pixel Leica binocular microscope. Pyramidal neurons and astrocyte counts, and level of oxidative stress were quantified using the software, image-j.

Data collected was analysed as mean±SEM employing one-way analysis of variance (ANOVA) followed by Tukey Post-hoc for multiple comparison using the GraphPad prism (San Diego, California, USA) version 7.02 at $p < 0.05$.

RESULTS

The escape latency (swimming time) of the AQ group was significantly higher than the CT, AS and AS+AQ groups. The AS and the AS+AQ groups also had a higher swimming time than the CT but not statistically significant (Figure 1). The swimming distance covered by the rats

in the AQ group was significantly higher compared with the CT, AS and AS+AQ groups. However, the AS+AQ group had a significantly higher swimming distance covered compared with the CT group (Figure 2).

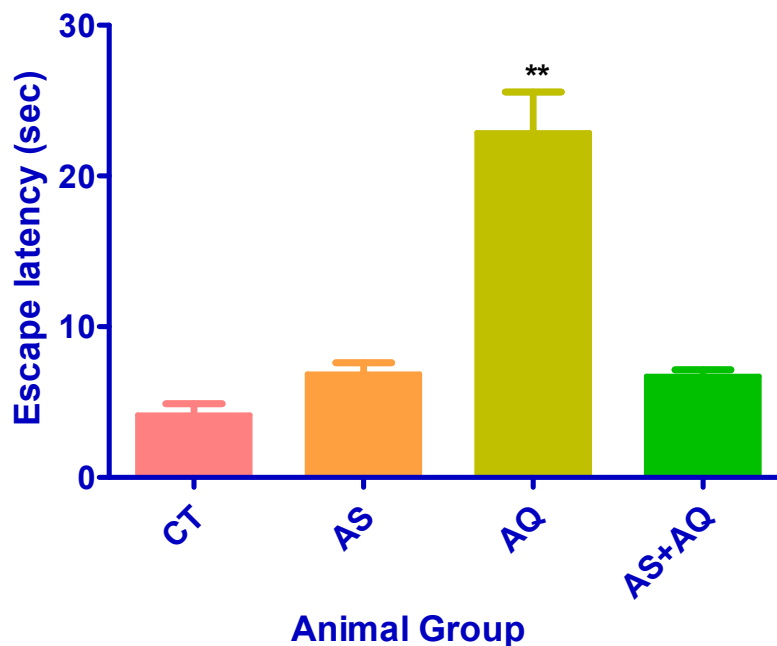


Figure 1. Sub-acute evaluation of the Swimming time in seconds. (probe trial) of animals treated with AS, AQ and AS+AQ. Means ± S.E.M. n = 10, $p < 0.05$. CT-Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. ** $P < 0.01$ compared with control and other groups.

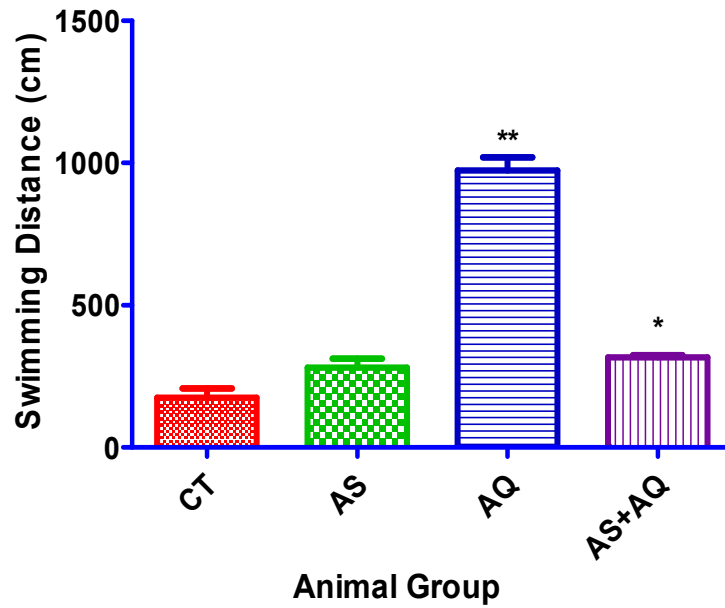


Figure 2. Sub-acute evaluation of the Swimming distance in cm (probe trial) of animals treated with AS, AQ and AS+AQ. Means \pm S.E.M. n = 10, $p < 0.05$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. * $P < 0.05$ compared with the control. ** $P < 0.01$ compared with the control and other groups.

There was no significant difference in the frequency of entering the escape platform

quadrant when the escape platform has been removed (probe trial) in all the groups (Figure 3).

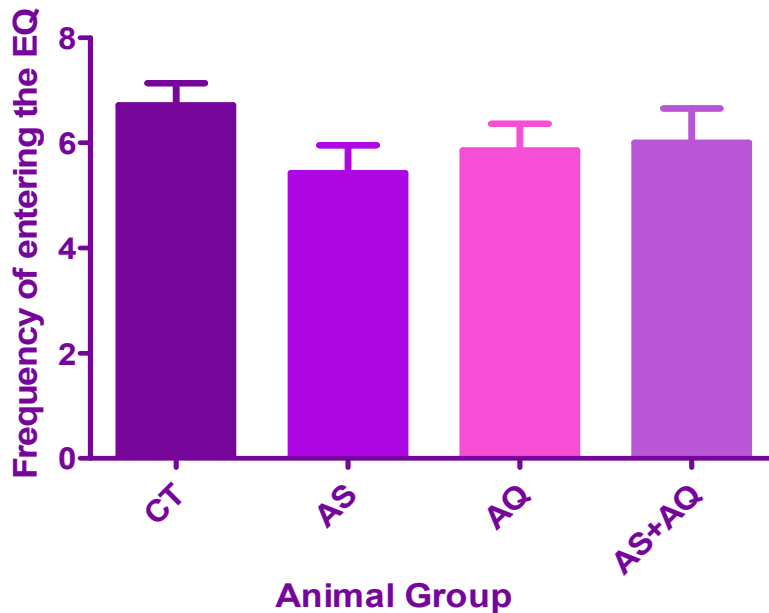


Figure 3. Sub-acute evaluation of the Frequency of entering the escape quadrant (EQ) of rats treated with AS, AQ and AS+AQ. Means \pm S.E.M. n=10, $p > 0.05$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. $P > 0.05$.

Table 1. Sub-acute evaluation of haematological parameters of rats treated with AS, AQ and AS+AQ

Blood Parameter	Group			
	CT	AS	AQ	AS+AQ
RBC ($10^6/\mu\text{L}$)	6.90±0.31	7.00±0.33	8.30±0.13*	7.40±0.22
WBC ($10^3/\mu\text{L}$)	9.10±0.90	10.40±1.42	18.90±1.99*	8.00±0.59
PLAT (10^3)	654.00±43.48	668.00±86.14	647.80±75.17	713.80±67.16
Hb (g/dl)	13.60±0.33	13.90±0.43	16.00±0.37*	14.70±0.55

Values are expressed as Mean ± SEM. n= 10, p<0.05. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. RBC: red blood cell, WBC: white blood cell, PLAT: platelet, Hb: haemoglobin, PCV: packed cell volume. *p<0.05 compared with the control.

PCV (%)	45.80±1.36	46.20±1.36	57.00±1.30*	50.80±1.36
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Histological evaluation of the hippocampal Cornu Ammonis 1 (CA 1) showed significant decrease in the pyramidal neurons of the AS and AQ - treated groups compared with the CT and AS+AQ groups (Figures 4 and 5). The

pyramidal neurons in the AQ and AS groups also showed severe pyknosis, with mild pyknosis seen in the AS+AQ-treated group compared with the CT group (Figure 4).

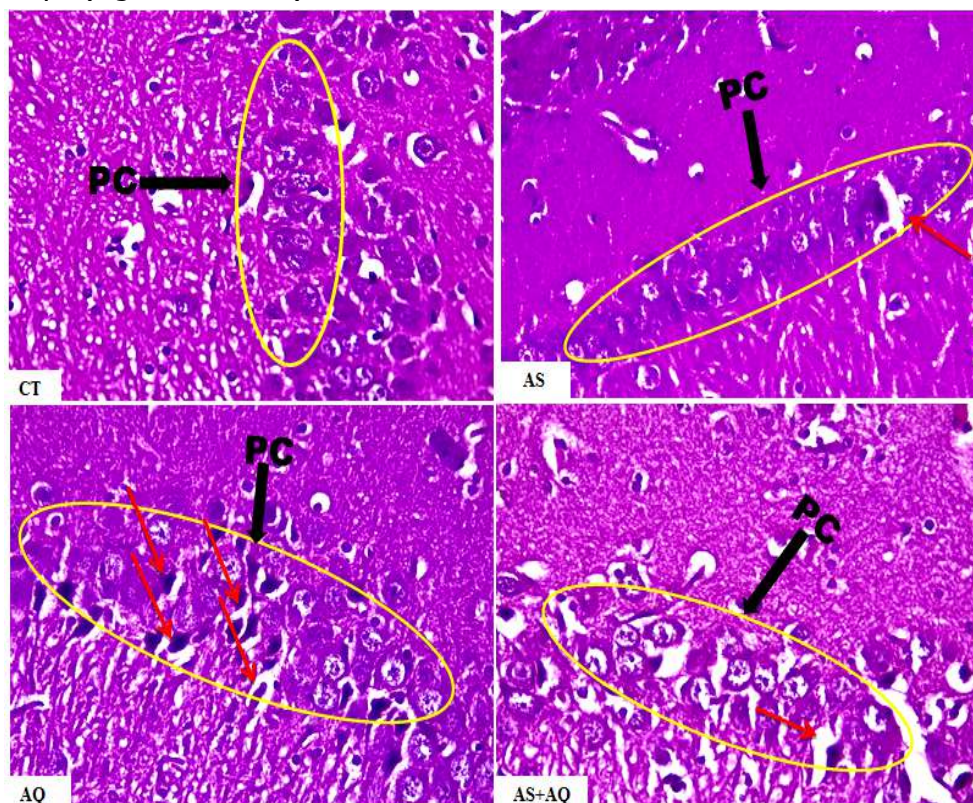


Figure 4. Sub-acute evaluation of the hippocampal CA 1 (Cornu ammonis 1) of rats treated with AS, AQ and AS+AQ. H&E (X400). CT= control group, AS= artesunate group, AQ= amodiaquine group, AS+AQ= artesunate-amodiaquine combination group. PC= Pyramidal cells. Red arrows: pyknotic pyramidal cells.

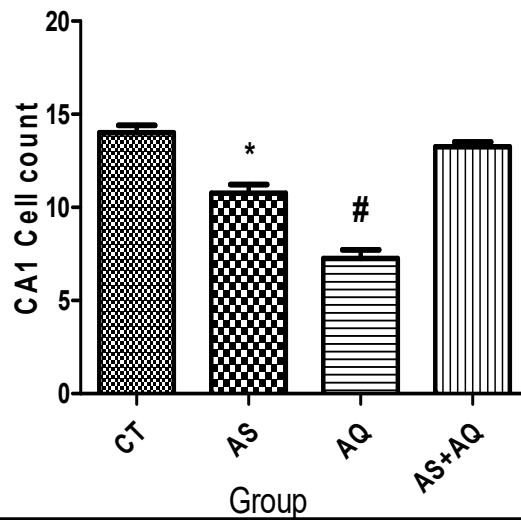


Figure 5. Sub-acute evaluation of the hippocampal CA 1 pyramidal neurons of rats treated with AS, AQ and AS+AQ. Means ± S.E.M. n = 5, p<0.05. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. *p<0.05 compared with the CT; #p<0.05 compared with the CT and AS+AQ groups.

Increased GFAP expression was observed in the hippocampus of AS and AQ-treated rats as indicated by increased astrocyte population

compared with the CT and AS+AQ groups at p<0.05 (figures 6 and 7).

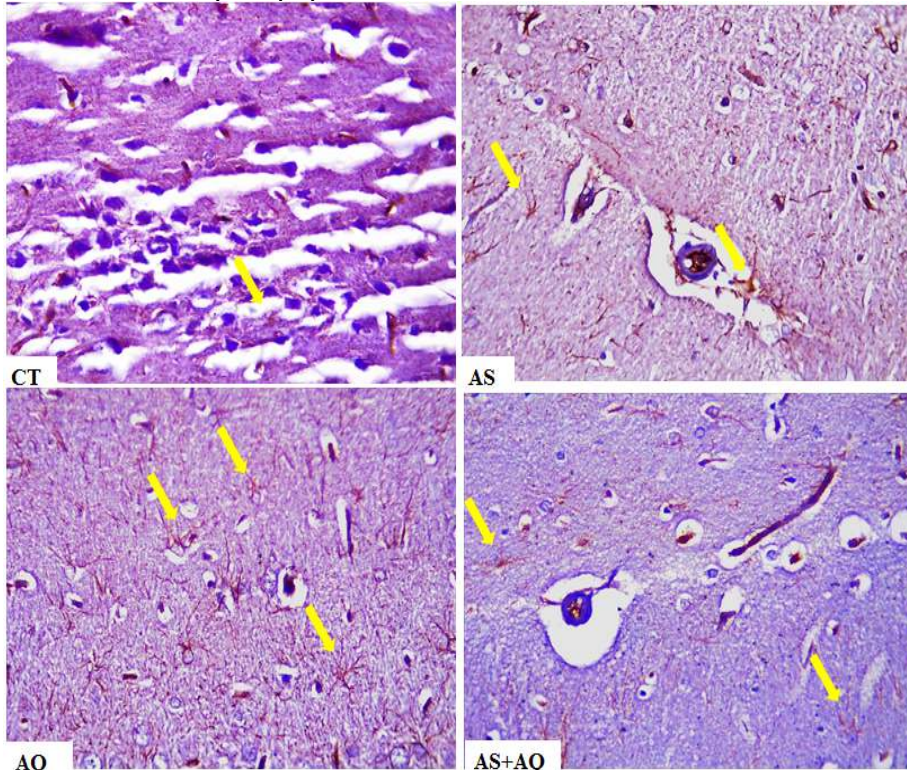


Figure 6. Sub-acute evaluation of astrocytes (yellow arrows) in the hippocampus of rats treated with AS, AQ and AS+AQ. GFAP (X400). CT= control group, AS= artesunate group, AQ = amodiaquine group, AS+AQ= artesunate-amodiaquine combination group.

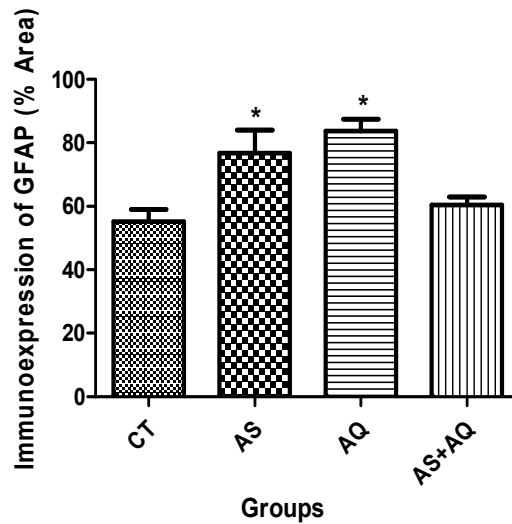


Figure 7: Sub-acute evaluation of astrocytes population in the hippocampus of rats treated with AS, AQ and AS+AQ. Means \pm S.E.M. n = 5, at $p < 0.05$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. * $p < 0.05$ compared with the CT and AS+AQ.

A higher expression of iNOS was seen in the hippocampus of the AS, AQ and AS+AQ-treated

group compared with the CT group at $p < 0.05$ (Figures 8 and 9).

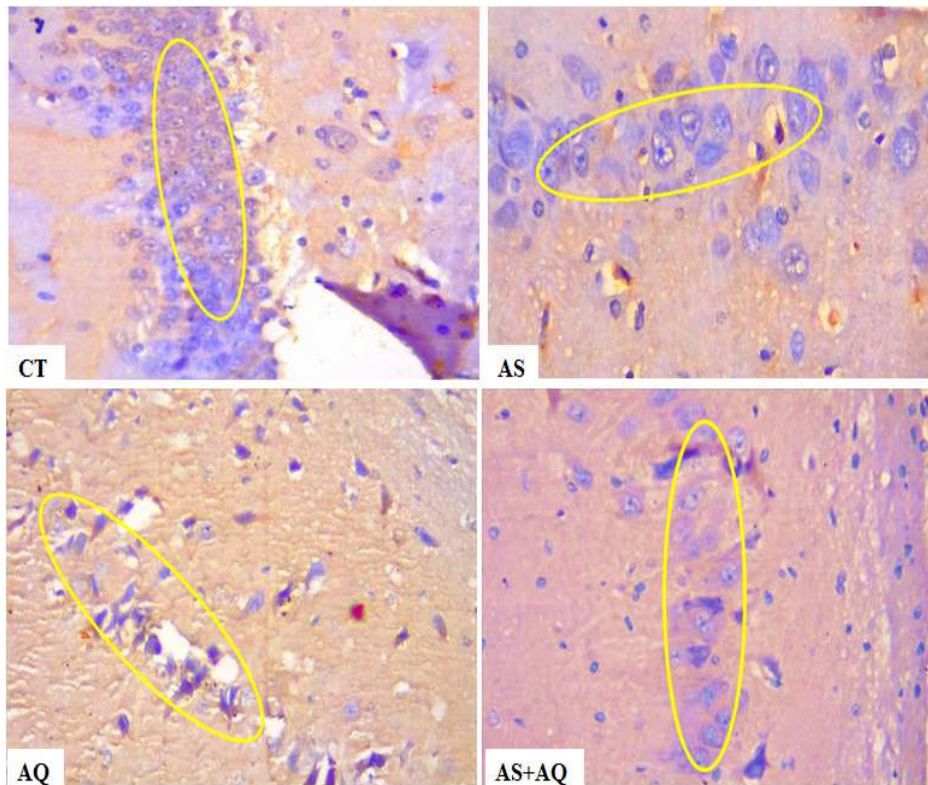


Figure 8: Sub-acute evaluation of the level of oxidative stress in the hippocampal CA 1 rats treated with AS, AQ and AS+AQ. iNOS (X400). CT= control group, AS= artesunate group, AQ= amodiaquine group, AS+AQ= artesunate-amodiaquine combination group. Yellow rings = Cornu Ammonis 1 neurons.

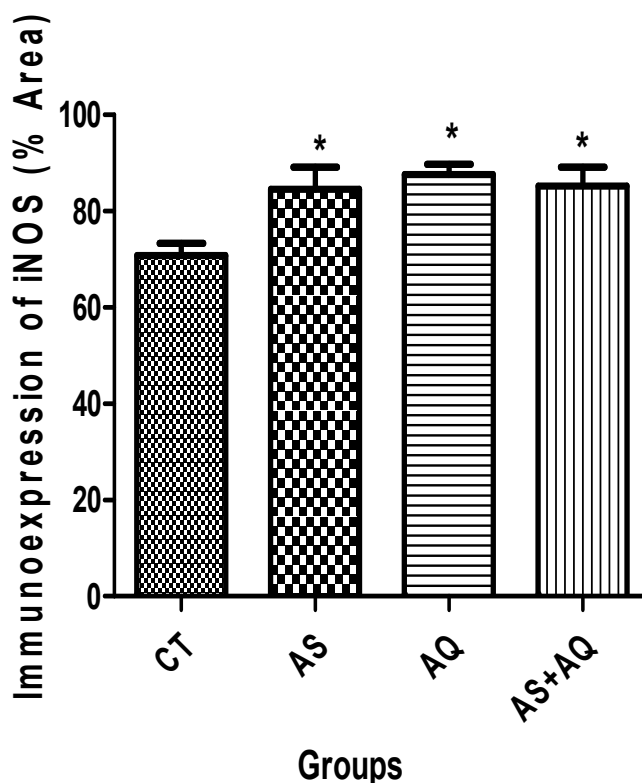


Figure 9. Sub-acute evaluation of the level of oxidative stress in the hippocampal CA 1 rats treated with AS, AQ and AS+AQ. Means \pm S.E.M. n = 5 at P<0.05. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. *P<0.05 compared with the CT group.

DISCUSSION

The significant increase in escape latency observed with the AQ group compared with the CT and the AS and AS+AQ groups is an indication that AQ retarded the spatial memory of the animals compared to AS and the AS+AQ groups which did not show significant reduction in the spatial memory of the rats compared to AS and the AS+AQ groups which did not show significant reduction in the spatial memory of the rats. Also, the insignificant variations in the frequencies of entering the escape quadrant between the experimental groups (AS, AQ and AS+AQ) and the control (CT) group and within the experimental groups suggests that AS and AQ separately and in combination did not have significant effect on the cognition of the animals. This finding is in line with Ekong *et al.* (2009) who reported in the acute study, that

artesianate may not be harmful at its recommended dose and may not affect behavior. They also reported that artesunate-amodiaquine combination showed no significant change in behaviour of rats (Ekong *et al.*, 2009). It also agrees partly with the findings of Onalapo *et al.* (2013) who reported a reduction in spatial memory scores in animals that received AQ. However, it disagrees with the same authors, Ekong *et al.* (2009) and Onalapo *et al.* (2013) who reported that AS also caused reduction in spatial memory. However, the variation in the findings of the two studies may be associated with the difference in the type of animals studied. The significant increased level of RBC and WBC recorded in the AQ group suggests that AQ boosted these blood cells probably by

stimulating the haemopoietic stem cells in the bone marrow to actively manufacture more blood cells. These blood cells also increased slightly in the AS and AS+AQ groups but not significantly different from that of the CT group. This suggests that none of the drugs reduced the blood cell counts. In a similar vein, the significant increase in the Hb and PCV levels in the AQ group coupled with their slight increase in the AS and AS+AQ groups compared with the CT group further emphasized that AQ, and to some extent AS and AS+AQ boosted blood cell and volume and did not cause anaemia. This finding also indicates that there was positive variation between RBC level and the Hb level. Hb is an oxygen carrying protein in the RBC, and usually, as the quantity of RBC increases, so does the level of Hb (Sembulingam and Sembulingam, 2009). It is also partly in line with Utoh-Nedusa *et al.*, (2009) who reported that dihydroartemisinin significantly elevated the packed cell volume, the total white cell count and percentage neutrophil count in rats. It also conforms with Aprioku and Obianime (2011) who also reported that artesunate, dihydroartemisinin, and artemether significantly and dose-dependently increased white blood cell count and that of Agomo *et al.* (2008) who reported that the mean of all the blood parameters were within the normal limits following artesunate/mefloquine treatment of uncomplicated falciparum malaria while there was slight increase in white blood cell count. The concordances of the present findings with the previous findings may be associated with the similarity in dosages of drugs administered and duration of study while the discrepancies between the current findings and some of the previous studies may be due to higher drug dosages, longer duration of study and even different species of animals used as reported in some of those studies.

The significant decrease in the number of hippocampal pyramidal cells (CA1) in the AS and AQ groups coupled with pyknotic cells in the AS, AQ and AS+AQ groups is a strong

indication that AQ and AS caused death of CA1 cells. This may be hinged on the oxidative stress cum damage prompted by the presence of significant expression of inducible nitric oxide synthase. In the hippocampus, pyramidal cells process sensory and motor cues to form a cognitive map encoding spatial, contextual, and emotional information, which they transmit throughout the brain (Graves *et al.*, 2013; Preston and Eichenbaum, 2013). Pyknosis of these cells may reduce the spatial memory of the animals as observed in the neurobehavioural test of this study.

Also, there was significantly increased astrocytes population in the AS and AQ groups compared with CT and AS+AQ groups, suggesting astrogliosis, an indication of neuronal damage or injury and may alter the hippocampal function due to possible alteration in the uptake of neurotransmitter by neurons. Astrocytes had been earlier described as neuroprotective cells which usually proliferate and increase in number during injury to the central nervous system in order to fill the injury sites. This process helps to heal and recover neurons; thus, with this action, they are called reactive astrocytes (Peter *et al.*, 1998 and Ekanem *et al.* 2009). Abbas and Nelson (2004) also reported that the presence of reactive astrocytes is an indication of early signs of neuronal cell loss, an indication of pathologic process. Findings from this study is in consonance with that of Udoh *et al.* (2014), who reported that Artesunate/Mefloquine combination (Artequin) and Mefloquine caused large and dense populations of astrocytes and astrocytes' processes in the hippocampus of adult Wistar rats. This, according to the authors may alter neuronal environment and impair the uptake of neurotransmitter thus altering the hippocampal function. This again further buttress the loss of spatial memory recorded against the AQ group in the Morris water maze test of spatial memory (a function of the hippocampus) in this study. Furthermore, the hippocampi of AS, AQ and AS+AQ groups had significantly higher expression of iNOS

compared to the CT, a strong indication that the drugs separately and combined, generated nitric oxide free radicals which caused oxidative stress in the hippocampus of the exposed animals and might be one of the causes of neuronal damage and death observed in the previous slides. Oxidative stress had earlier been implicated in tissue damage (Mayes, 2000) and defective function of the brain (Singh, 2013).

In conclusion, sub-acute evaluation of adult Wistar rats treated with artesunate (AS),

amodiaquine (AQ) and their combination (AS+AQ) showed that AQ reduced the spatial memory of the animals and increased the level of most of the blood cell parameters of the animals; AS, AQ and AS+AQ caused pyknosis of pyramidal CA1 cells in the hippocampus; AS and AQ caused astrogliosis in the hippocampus; and AS, AQ and AS+AQ caused oxidative stress in the hippocampus of the rats. From the above study, artesunate and amodiaquine and their combination are considered to have some deleterious effects in the hippocampus of adult Wistar rat following sub-acute studies.

Declaration of interest: The authors declare that there is no conflict of interest in this study

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