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Effects of Six Artemisinin-Based Combination Therapies On Blood Glucose, Pancreatic Histology and Insulin Immunolocalization: An Experimental Malaria Study.

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ABSTRACT
Acute pancreatitis has been reported during and after malaria treatment. This study characterized the effects of six artemisinin-based combination therapies (ACTs) on the blood glucose, pancreas histology and insulin immunolocalization in a curative malaria model. Forty male Swiss adult mice were divided into 8 groups of 5 animals each. Murine malaria was induced in groups 2 – 8 via intraperitoneal injection of 0.2 ml containing 1 x 10^6 *Plasmodium berghei ANKA* parasites. Groups 1 and 2 representing negative and positive controls (NC and PC), received 5 ml/kg distilled water only, groups 3 to 8 received oral therapeutic doses of 5.7 mg of artesunate-amodiaquine (AA), 6.43 mg artesunate-mefloquine (AM), 25.36 mg artesunate-sulfadoxine-pyramethamine (ASP), 12.5 mg artemisinin-piperaquine (AP), 5.14 mg dihydroartemisinin–piperaquine (DP) and 8 mg arthemeter-lumefantrine (AL) respectively standard regimen of 2-3 days, and 24 hours after the last treatment, animals were sacrificed under chloroform inhalation. The pancreases were carefully harvested, rinsed in normal saline and fixed in 10 % buffered formalin for tissue processing. Final parasitemia, blood glucose levels, pancreas histology and insulin immunolocalization were determinedby standard protocols. Results showed all the ACTs were effective against hyperparasitemia except ASP, and AM appeared the most effective. Blood glucose was not significantly altered, and this correlated with strong insulin expression across all groups, there was mild edema and inflammation in the test groups compared to NC. In conclusion murine malaria and hyperparasitemia clearance with the six ACT may cause acute pancreatitis but does not alter blood glucose levels, and strongly demonstrated insulin expression.

Keywords: Malaria, Artemisinin-based combination therapies, ancreas, Insulin, Blood glucose

INTRODUCTION
Malaria is a life-threatening mosquito-borne blood disease caused by *Plasmodium* parasite and it is transmitted to humans through the bite of anopheles' mosquitoes [1]. The transmitted agent is a one-celled parasite called *Plasmodium*, and there are 5 species that cause malaria in humans of which *Plasmodium falciparum* poses the greatest threat and the others; *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* generally cause a milder form of malaria [2, 3]. Malaria is one of the common causes of acute febrile illness in endemic areas and can cause single or multi-organ dysfunction including acute renal failure, acute respiratory distress syndrome (ARDs), jaundice, myocarditis, hemolytic anemia and coma [4].

According to the WHO, there were 216 million cases of malaria in 2016 and estimated number of malaria death stood at 445, 000 in 2016 [1], and Nigeria suffers the greatest malaria burden of the disease with approximately 51 million cases and 207, 000 deaths reported annually (approximately 30% of the total malaria burden in Africa) while approximately 173 million are at risk of the infection [3].

WHO has recommended artemisinin-based combination therapies (ACTs) as first line treatment of uncomplicated *Plasmodium falciparum* [5-7]. Artemisinins are derived from a plant called Artemisia annua (sweet wormwood or sweet Annie), [8]. It was discovered in 1971 by a Chinese Scientist called Tu Youyou [9]. They are among the most potent anti-malaria agents; they are highly effective against nearly all asexual and sexual parasite stages [10-12]. They can kill parasites within minutes with a parasite reduction ratio of approximately 10,000 per erythrocytic cycle, resulting in rapid clinical responses [8, 13]. The role of artemisinin is to reduce the number of parasites within the first 3 days of infection, while the partner drugs eliminate the rest [7].

Artemether-lumefantrine is a fixed dose oral combination for testing uncomplicated *falciparum* malaria in adults and children [14]. Its excellent efficacy has been validated in multiple clinical trials [15]. Artemether-mefloquine has been widely used in SouthEast Asia; the recently developed fixed dose combination with tablet shows excellent efficacy and improvement in tolerability [16]. Piperaquine was developed as a replacement of chloroquine and is extensively used in China [15]. Several other ACTs such as artesunate amodiaquine, artesunate

sulfadoxine–primethamine and artesunate chloroprogunanil have been developed and are under clinical trials [17].

Malaria has been reported to affect the pancreas; it is a rare cause of pancreatitis [18-22]. The possible mechanism of pancreatitis in malaria is micro-vascular occlusion with resultant ischemia, activation of pancreatic enzymes and injury to the pancreas due to auto digestion [23-26].

However, the current treatment of malaria with different ACTs warrant investigation on blood sugar and pancreatic alteration as there is dearth of literature on this. This study investigated the effect of six ACTs on the blood glucose levels, pancreas histology and insulin immunolocalization in an experimental malaria model.

MATERIALS AND METHODS

Drug Acquisition

Six commercially available ACTs namely: (CAMOSUNATE® that is Artesunate-amodiaquine produced by FRONT PHARMACEUTICAL PLC., Anhui, China), Artequin™ that is Artesunate-mefloquine produced by Acino Pharma AG, Liesberg, Switzerland), (SIMCURE® that is Artesunate-sulfadoxine-pyrimethamine by Jiangsu Province, China), (ARTEQUICK® that is Artemisinin-piperaquine produced by Artepharm Co., Ltd., China), (P-ALAXIN™ that is Dihydroartemisinin-piperaquine produced by BLISS GVS PHARMA LTD., Maharashtra, India), (Coartem® that is Artemether-lumfantrine produced by Novartis Saglik, Base, Switzerland), were all purchased at a Pharmaceutical Store in Uyo metropolis.

Animal Handling and Care

Forty (40) male albino Swiss adult mice with weights ranging from 20 – 30 g were bred at the animal holdings of the Faculty of Basic Medical Sciences, University of Uyo, Uyo. The animals were kept in spacious cages with soft wood shavings (saw dust). They were exposed to 12 hours' light and 12 hours' day cycle at 22 - 24^{0}C. The animals were kept five (5) per cage to allow them a degree of freedom and were given access to food and water *ad libitum*. The cages were cleaned and the beddings changed on a daily basis. All animal experiments were performed in accordance with the National Institute of Health Guide for the care and use of laboratory animals [27].

Parasite Inoculation and Parasite Estimation

The *Plasmodium berghei* used was obtained commercially from the National Institute of Medical Research (NIMER), Yaba, Lagos, Nigeria. Thirty-five mice were inoculated intraperitoneally with 0.2 ml of infected blood from the donor mice containing about 1×10^6 *Plasmodium berghei*, parasitized erythrocytes per ml. This was prepared by determining both the percentage parasitemia and the erythrocytes count of the donor mouse and diluting the blood with isotonic saline in proportions indicated by both determinations [28]. Stained thick and thin blood smears were obtained from the tail vein of the mice onto clean frosted slides and were made and viewed under oil immersion at X100 magnification using a light microscope (Olympus CX31, Japan); thereafter digital photomicrographs were obtained using a digital microscopic camera (Amscope Digital Camera with Model number MU1000, China).

Experimental Design

The animals were kept for two weeks to acclimatize; their weights and blood glucose level were checked and were divided into 8 groups according to weight range.

Treatment Groupings	Dosage	Duration (days)
NC normal saline	5 ml/kg	3
Pb	1 x 10^6 parasites	3
Pb + AA	5.7 mg	3
Pb + AM	6.43 mg	3
Pb + ASP	25.36 mg	2
Pb + AP	12.5 mg	3
Pb + DP	5.14 mg	3
Pb + AL	8 mg	3

Calculation of Dosage: Each drug was administered based on the body weight of each animal using the formula = weight of rats/1000 x dose/stock.

Termination of Experiment: 24 hours after the last dosage, the blood glucose levels were checked. All the animals were sacrificed using humane killing method by anaesthetizing them in a chloroform chamber. The pancreas was harvested, rinsed in normal saline and fixed in a sample bottle containing 10% buffered formalin.

Blood Glucose Determination: The tails of the animals were pricked using lancet, the blood was obtained and used on the Fine Test glucometer® to determine the glucose level.

Histopathological assessment: A pancreas of one of the experimental animals from each group was removed after intracardiac perfusion, and fixed in 4% paraformaldehyde, and processed within 72hrs for light microscopy. The paraffinized tissue blocks were sectioned at 4 microns using a rotary microtome, and stained with Haematoxylin and Eosin (H&E), and insulin antibody expression (Insulin-Biogenix Lot: Am0291214), and thereafter examined under light microscope. The photomicrographs were obtained using the Olympus microscope attached to an Amscope digital camera (MU 1000 China).

Statistical Analysis

Data obtained from this study were analyzed using Graphpad 6 version 11 system package. Results were expressed as mean ± standard error of mean. One-way ANOVA and multiple comparison and t-test were

employed with the significance level of $P < 0.05$

RESULTS

Parasitemia of *Plasmodium berghei*-infected Male Albino Adult Swiss Mice: Clearance of parasites in all the test groups but was rapid and in the ASP treated group the parasite levels decreased tremendously as presented in Table 1.

Blood Glucose Levels Following Administration of Six ACTs to *Plasmodium berghei*-infected Male Albino Adult Swiss Mice: The blood glucose level was not statistically significantly at $p < 0.05$, the blood glucose level appeared within the normal range and this correlated with the immunohistochemical result as seen in Table 2.

Histological Findings: The histological observations are represented in Figs. 1 – 2, and they revealed normal to abnormal histoarchitecture of the pancreas and insulin localization respectively.

Figure 1: Parasitemia of *Plasmodium berghei*-infected Male Albino Adult Swiss Mice

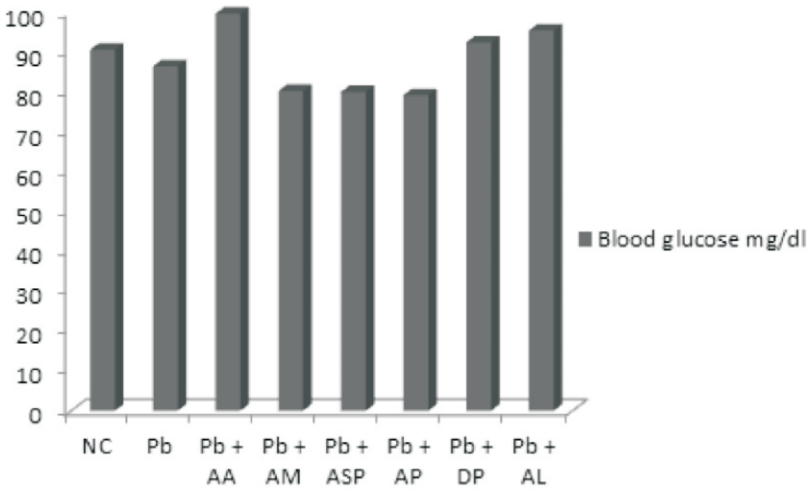

Figure 2: Blood Glucose Levels Following Administration of six ACTs to *Plasmodium berghei*-infected Male Albino Adult Swiss Mice

Table 1: Insulin Immunohistochemical Expression following Administration of six ACTs administration to *Plasmodium berghei*-infected Albino Adult Swiss Mice

Group	% of IHC	Intensity of IHC	Final Score	Expression
1	>60%	Strong	6	High
2	>60%	Strong	6	High
3	>60%	Strong	6	High
4	>60%	Strong	6	High
5	>60%	Strong	6	High
6	>60%	Strong	6	High
7	>60%	Strong	6	High
8	>60%	Strong	6	High

Scoring system for immunohistochemistry by Klien *et.al* (1999) [29]

Key:

% IHC	Intensity of IHC	Final Score	
0 = 0%	0 = no reaction	A + B	= range from 0 to 6
1 = <30%	1 = weak	0/6	= Negative Reaction
2 = 30-60%	2 = mild	1/6, 3/6, 3/6	= Low Expression
3 = > 60%	3 = strong	4/6, 5/6, 6/6	= High Expression

Histological Observation

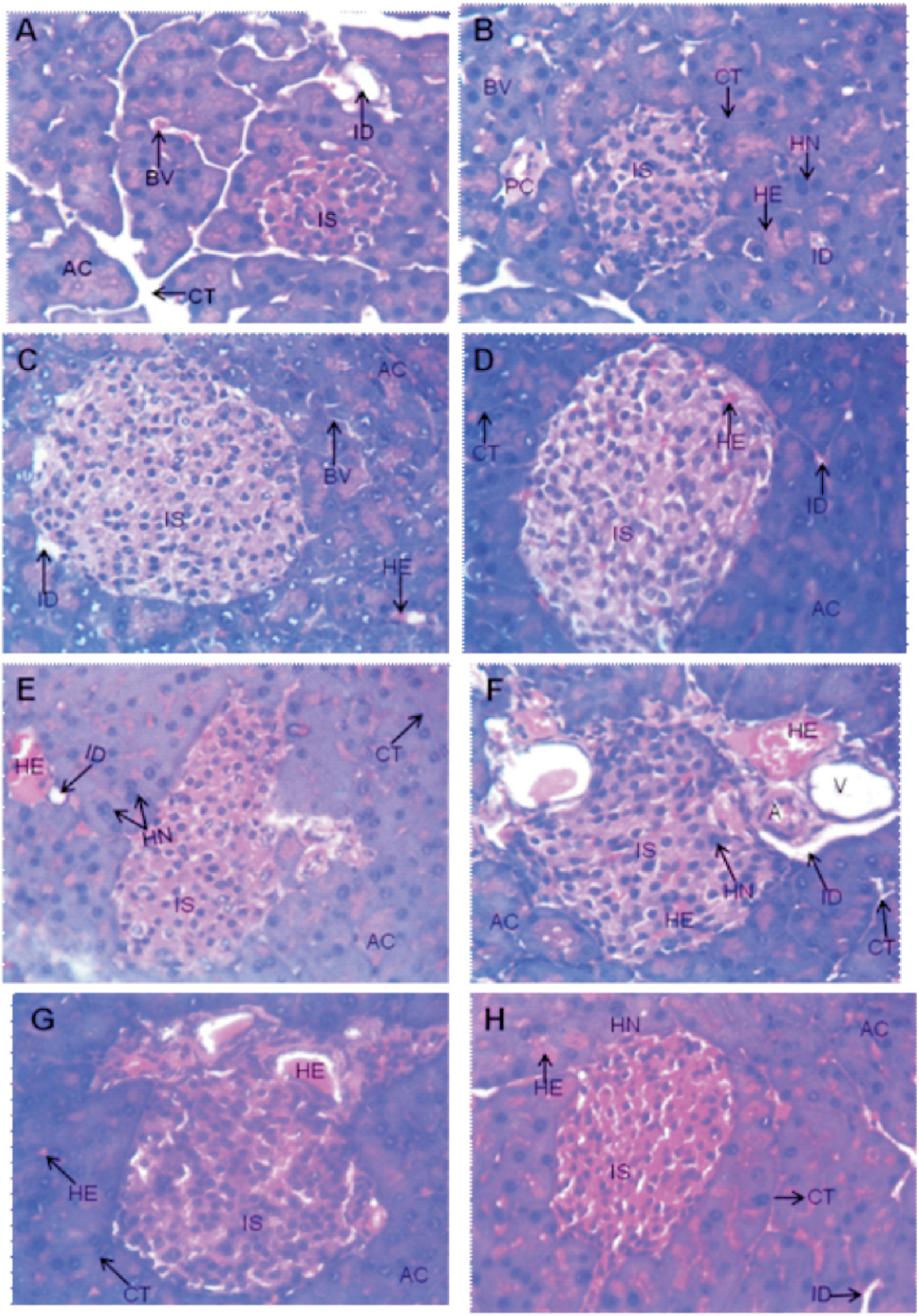

Figure 3: Effect of ACTs (B-H) on the cross section of Pancreatic Histology compared to control (A) (H&E x400)

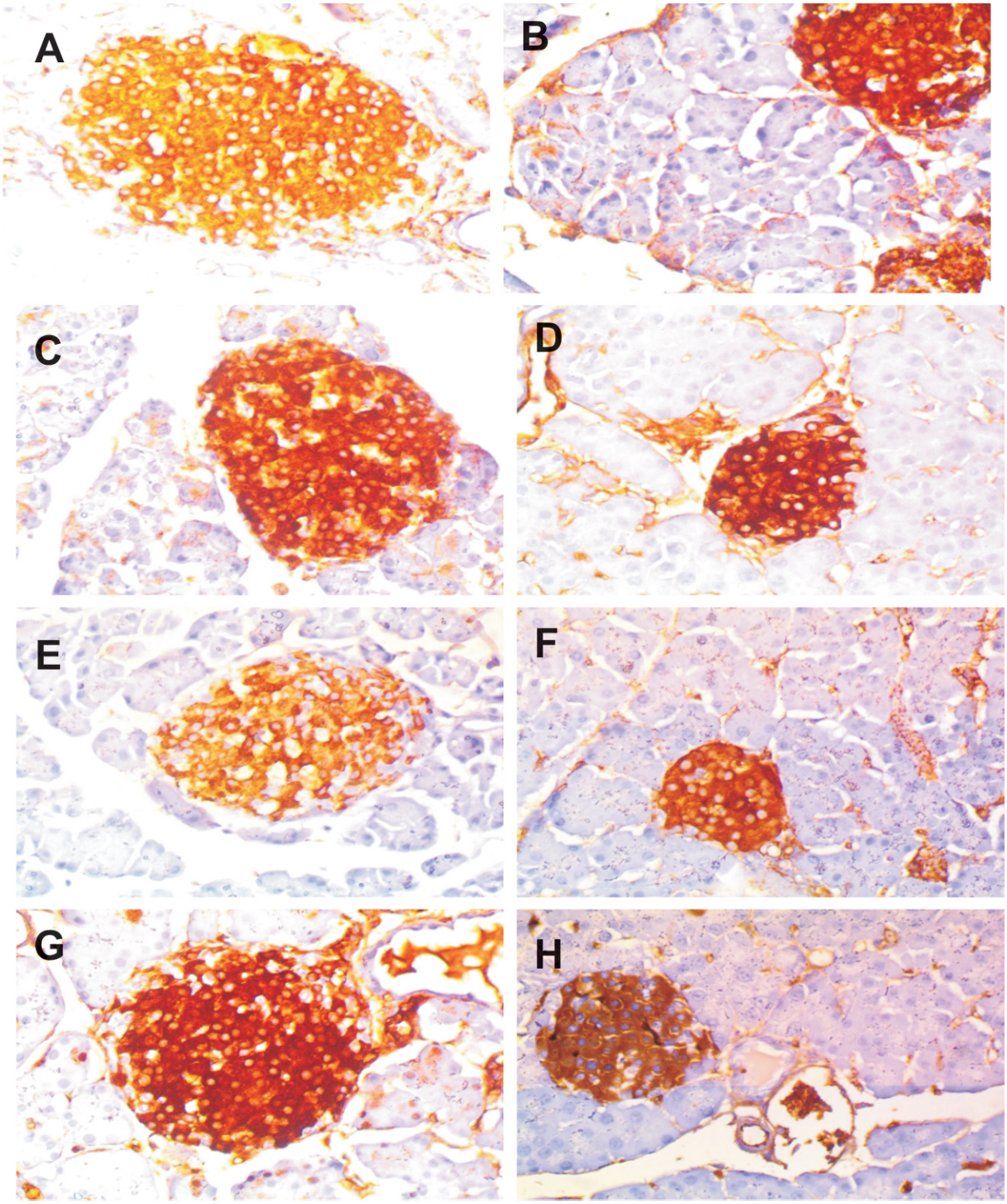

Figure 4: Effect of ACTs on pancreatic insulin immunolocalization in test groups (B-H) compared to control (A) (H&E x400)

DISCUSSION

Artemisinin-based combination therapies need continuous monitoring to keep track of any negative effects on various organs thus they deserve more scrutiny because of their widespread usage [30]. The energy level in severe malaria is depleted by 10% of normal ATP [31]. This depletion occurs due to insufficient oxygen reaching the mitochondria and it renders it unable to generate energy from oxidative phosphylation, therefore causing fatigue in patients suffering from malaria [32]. This study investigated the effect of six ACTs on the pancreas histology and insulin immunolocalization in an experimental malaria model.

Before the administration of ACTs, it was observed that the infected but untreated animals exhibited symptoms of malaria which included; weight loss, loss of appetite, decreased locomotive activity and fatigue compared to NC [3]. NC showed no parasite while PC had an increased final parasitemia. Parasite clearance was demonstrated in all, but one of the ACT brands that is ASP. AM treated group had the highest efficacy, followed by DP, AP, AA, AL, while ASP treated had the least efficacy. Georgewill and Ebong [33] had earlier reported that AA has the highest efficacy followed by DP, ASP, AM and AL.

Nsonwu-Anyanwu [34], demonstrated that hyperparasitemia is associated with high glucose requirements by malaria parasites, which can lead to clinical hypoglycaemia. The blood glucose levels for groups 1 - 8 were within the range of 80.00 – 99.60 mg/dl, and were not statistically significant, which correlated with the immunohistochemical evaluation seen in Fig. 4 (A-H), in which highly expressed insulin immunopositive cells were found in greater density in the central part of the islets of Langerhans in the form of dark brown granules in all the controls compared to the treated groups, therefore indicating normal secretion and production of insulin. Philips [35] had reported that antimalarial drugs such as amodiaquine, mefloquine and halofantrine have no direct effect on insulin secretion. IL-1 and 6 released during malaria infection can cause islet cell hyperplasia resulting in enlargement of pancreatic islets and subsequent increase in insulin expression [36]. This can be seen demonstrated in photomicrographs in Figure 4.

Histological examination of NC showed normal histological structures, and the islets of Langerhans appeared as non-capsulated pale stained oval areas inside the lobules which were formed of groups of cells arranged in irregular, branching and anastomosing cords [***37]. P***ancreatic tissues to have been reported show numerous parasitized red blood cells in the capillaries [36]. These parasitized red blood cells can cause increased blood viscosity and decrease in red blood cell membrane deformity. These circulatory disturbances can lead to tissue hypoxia, release of pancreatic acinar enzymes, impaired capillary and venous drainage, which in turn can lead to haemorrhagic pancreatic necrosis [38]. There was vascular congestion and damage of the blood vessels which resulted in interstitial haemorrhage in the parenchyma and islets of Langerhans. The acinar cells appear congested and the nuclei tend to be hypertrophic indicating edema and mild inflammation which is the basic histological morphology of acute pancreatitis [39]. In AA, AM, ASP, DP and AL groups, the acinar cells were congested with hyperplastic cells and mild haemorrhage while in the AP treated group; there was severe haemorrhage with hypertrophic nuclei. This result is in accordance with [40, 41], who demonstrated that malaria caused the accumulation of parasitized erythrocytes inducing thrombosis and infarction. Pancreatic tissues revealed acute inflammatory reactions of acini, islets cells and interlobular ducts.

CONCLUSION

It is concluded from this present study which is the first to investigate the therapeutic doses of six different ACTs on the blood glucose levels, pancreas histology and insulin expression for a standard regimen of 2 – 3 days, that ACTs do not significantly alter blood glucose levels during murine malaria infection, though ASP and AP treated groups had lower glucose levels with mild pancreatitis, however strong insulin expression was observed across all ACT-treated groups.

CONFLICT OF INTEREST

None is declared

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