Haematological Indices in Artemisinin, Vitamins C and E Co-Administration to Malaria infested Mice

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Website
http://ijfmi.com/

Doi:
https://doi.org/10.21816/ijfmi.v4i2

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ABSTRACT

Introduction: With Artemisinin-based combination treatments (ACTs) reportedly the first line remedy for uncomplicated cases of *falciparum* malaria in several countries, over 40 malaria-endemic nations in sub-sahara Africa now adopts its use as such. This study examined the effect(s) of Antioxidant Vitamins co-administration with ACTs on haematological variables [Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Eosinophil Count (EC), Monocyte Counts (MC), Packed Cell Volume (PCV) and Lymphocyte Count (LC)] of malaria-induced, immune-compromised mice.

Materials and Methods: Seventy (n = 70) adult male (20g- 35g) albino mice (Swiss strain) were grouped into seven (7) of ten (10) rats per group. With Group 1 receiving standard diets (control), Group 2 was inoculated with *Plasmodium. berghei* and left untreated (Negative control group); whereas, Groups 3 - 6 respectively received (following inoculation with *P. berghei*); 150mg/kg body weight of Vitamin C, 150mg/kg body weight of Vitamin E, 56mg/kg body weight of Artemisinin, 56mg/kg body weight of Artemisinin + 150mg/kg of Vitamin C, and 56mg/kg body weight of Artemisinin + 150mg/kg of Vitamin E. Following treatment period (28 days), animals were fasted overnight and sacrificed by cervical dislocation. Blood samples were obtained by cardiac puncture, analysed and compared for changes in haematological variables.

Results: Upon comparison between groups Analysis of Variance (ANOVA) returned a statistically significant difference for MCV, MCH, MCHC and EC, MC, MCHC of Groups 2 and 3 mice respectively; even though group 4 showed a statistically insignificant difference in PCV, EC, MC, and MCHC upon comparison with control. Study also found mean values of PCV, LC, MCV, MCH, and MCHC to be statistically insignificant in ACT co-administration with anti-oxidant vitamins.

Conclusion: We recommend vitamins C and E co-administration with ACTs to aid recovery in malaria sufferers.

Key words: Malaria, Haematological variables, Anti-oxidant Vitamins

INTRODUCTION

Malaria, a mosquito borne disease is a dreadful infection from a single-celled parasite of the genus, *Plasmodium.*[1] Through their bites, the Anophele mosquitoes transmit these parasites from an infected blood of a sufferer to someone else. Malaria is characterized by periodic bouts of severe chills and high fever; serious cases of which can result in death if left untreated. Annually, over a million cases of reported malaria lead to death across the globe, with most of them occurring in Africa [1]. Studies have shown that the multiplications of the parasites in human blood cells produce the recurrent attacks of fever and chills that are symptoms of the disease.[2]

Several Haematological changes have been associated with malaria infection. These changes reportedly affects the red blood cells, leukocytes and thrombocytes [3, 4] Studies have also report significantly lower platelets, WBCs, lymphocytes, eosinophils, RBCs and Hb levels in malaria infested individuals with monocyte and neutrophil counts proving to be significantly higher in comparison with non-malaria infected subjects[5] Again, individuals with platelet counts < 150,000/µL have been asserted to be 12-15 times more likely to have malaria infection than persons with platelet counts > 150,000/Ml.[6]

The control of malaria has been challenged by increasing resistance of *P. falciparum* to antimalarial drugs, particularly the chloroquine and sulfadoxine pyrimethamine variants. This has hugely revolutionized the antimalarial treatment recommendations,[6] causing the National Malaria Control Programme to recommended the use of Artemisinin-based Combination Treatments ACTs for the management of uncomplicated malaria cases across the globe. However, for reasons of increased effectiveness, low resistance, few side effects, and high tolerance, [7] the use of several brands of ACTs has become famous amongst clinicians, and often recommended to their clients who suffer chronic malaria infection, [8]

Currently, the WHO recommends ACTs for the first-line treatment to remedying high resistance malaria infections. In a study carried out by Godswill and Olawale, (2016), it was reported that co-administration of anti-oxidant vitamins with ACTs may reduced plasma ALT and AST activities in malaria infested mice, suggestive that co-administration of vitamin C might suppress the progressiveness of malaria parasite in infested mice.[9, 10] To this point, current study was designed to investigate the effect of Antioxidant Vitamins co-administration with ACTs on hematological variables of malaria infested mice. Specifically, study attempted to examine the changes in hematological variables for malaria infested, untreated mice, against those of healthy mice. Study also ascertained hematological changes in malaria infested, ACTs and antioxidant Vitamins (C and E) treated mice

MATERIALS

Scope of Study

Study was designed to be ex-vivo, adopting albino mice (Swiss strain) as experimental mode. The reason for choosing mice was due to the invasive approach; more so that inoculation of malaria with *P. Berghei* could not have been possible in humans. Study was conducted with at the animal house of the Faculty of Basic Medical Sciences, Delta State University, Abraka, Delta State, Nigeria; and was limited to investigating the impact of ACT, Vitamins C and E co-administrations on haematological variables of malaria-induced (immune-compromised) mice.

Study Design

Study adopted of a total of seventy (n = 70) adult male (20g- 35g) albino mice of the swiss strain. These mice were grouped into seven (7) of ten (10) rats per group; Group 1 received a standard rat diet ad libitum (control), Group 2 was inoculated with *Plasmodium berghei* and left untreated

(Negative control); whereas, Groups 3 - 6 respectively got (following inoculation with *P. berghei*); 150mg/kg body weight of Vitamin C, 150mg/kg body weight of Vitamin E, 56mg/kg body weight of Artemisinin, 56mg/kg body weight of Artemisinin + 150mg/kg of Vitamin C, and 56mg/kg body weight of Artemisinin + 150mg/kg of Vitamin E.

METHODOLOGY

Inoculation with *Plasmodium Berghei*

Malaria parasites, *plasmodium berghei* were obtained from the Nigerian Institute of Medical Research (NMR), Yaba, Lagos. Mice in the experimental group were infected by obtaining parasitized blood (3-4 drops) from the cut tail tip of the infected mice (donor). Next, 0.1ml of the collected infected blood was diluted in 0.9ml of phosphate buffer of pH 7.2 and the mice were inoculated with 0.1ml of the parasitized blood intraperitoneally as described by David *et al.,* (2010). [11] This contained about twelve thousand (12000) parasites.

Determination of parasitaemia

Parasitaemia was confirmed by preparing a thin blood film from blood obtained from the cut tail of the infected mice. This was stained with Giesma stain and viewed under the microscope. Determination was then done by counting at least, three fields per slide with 200 TWBC per field; [12]

$$\text{Parasites/}\mu\text{L of blood} = \frac{\text{No. of parasites x TWBC count/}\mu\text{L}}{200\text{(total leucocytes counted)}}$$

Drug Preparation and Administration

Coartem

The antimalarial drug Coartem; of the Artemether / Lumefantrine variant, Vitamin C (Ascorbic acid) and Vitamin E (α-tocopherol) were obtained from local Pharmacy store in Abraka, Delta State, Nigeria. The 6 tablets containing 80/480mg/kg of both active ingredients (Atemether/Lumefantrine) were meshed into powder form and further homogenized in 150ml of distilled water (H_20). The homogenate was then allowed to stand for 24 hours after a series of periodic stirring. The mixture was collected in a clean container and preserved in a refrigerator at minimum cool temperature. Using the orogastric canula, 56mg/kg (0.25ml) of Artemether/Lumefantrine was administered morning and evening orally between 8:00am and 4:00pm for 3 days.

Vitamin C

Five hundred milligram (500mg) of Vitamin C tablets were obtained from Rio Pharmacy in Abraka, Delta State, Nigeria. Each tablet (500mg) was dissolved in 100ml of distilled water, with the mixture centrifuged to obtain clear Vitamin C solution. This was then administered orally at a dose of 150mg/kg twice daily with orogastric canula.

Vitamin E

The tablets were dissolved in distilled water, at a dose of 150mg/kg and administered orally via an orogastric canula twice daily.

Sacrifice and Samples Collection

At the end of inoculation and treatment, animals were fasted overnight and sacrificed by cervical dislocation. Blood samples were obtained by cardiac puncture and placed in an EDTA sample container for haematological analysis.

Analysis of Haematological Parameters

Determination of Packed Cell Volume (PCV)

Blood was collected and filled with heparinised capillary tube. The tube with the blood was centrifuged at a speed of 11000 revolutions per minute (rpm) for 5 minutes. RBCs packed at the bottom forms the packed cell volume with the plasma remaining above. Centrifuge was then allowed to stop automatically before reading the PCV values with the microhematocrit reader.

Determination of Total White Blood Cell (TWBC) Count

This was done in line with standard technique as described by Ramnick, (2003). [13] The blood sample was diluted 1:5 with Turks solution which is 1% glacial acetic acid. With the aid of a capillary tube, the diluted sample was loaded into an improved Neuber counting chamber and the TWBC was counted from appropriate squares in the chamber using a microscope.

Determination of Total Red Blood Cell (TRBC) Count

Red blood cell count was determined from standard methods described by Chesbrough, (2000). [14] The blood sample was diluted to 1:20 with Hayen's fluid ($HgCl_2$ 0.05g; Na_2SO_4 2.5g; NaCl 5g in 100ml of water). The diluted sample was loaded into the improved Neuber counting chamber with the aid of a Pasteur pipette. RBC was then counted from appropriate squares in the chamber using a microscope.

Determination of Haemoglobin Concentration

Two test tubes were labelled Test and Blank. Five milliliters (5ml) of haemoglobin reagent was added to each test tube. 200µ (0.02ml) of plasma sample was then added to the test tube labelled Test and mixed properly. The solution in the test tube was then allowed to stand for 3 min at room temperature. The absorbance of the mixture was read with a spectrophotometer at 545nm.

Differential White Blood Cell Count

With the aid of a pasture pipette, a drop of blood was placed on a clean slide and a thin blood film was made from it. The thin film was then allowed to air dry. Next, it was stained with Leishman stain and air dryed. A drop of oil immersion was placed on a stained portion of the slide and a cover slip was placed on top the oil immersion. The film was then viewed under the microscope with cells are identified and counted per field with 40x objective lens using the differential WBC counter.

Determination of platelet count

Platelet count was made by measuring 380µl (0.38 ml) of filtered ammonium oxalate diluting fluid into a small test tube. 20 µl (0.02 ml) of well-mixed anticoagulated blood was added and mixed thoroughly. The improved Neubaeur counting chamber was filled with the well-mixed sample and left undisturbed for 20 minutes. The underside of the chamber was dried with cotton wool and viewed under the microscope with 40x objective to count platelets that appeared as small bright fragments (refractile).

Ethical Clearance

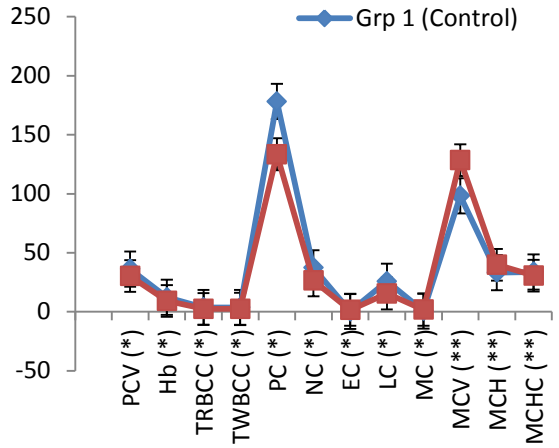
Prior to investigation, Ethical clearance was obtained from the Research and Ethics Committee of the Faculty of Basic Medical Sciences, College of Health Sciences, Delta State University, Abraka, Delta State. Animals were handled in accordance with protocols approved by the institutional animal ethics committee (IAEC), as adopted by the Faculty of Basic Medical Sciences, Delta State University, Abrake, Nigeria.

Statistical Analysis

Results obtained from the study were expressed as Mean ± SEM (Standard Error of Mean). With P-value of less than 0.05 ($p < 0.05$) considered to be statistically significant, a one-way analysis of variance (ANOVA) was used to determine the mean differences for variables between groups.

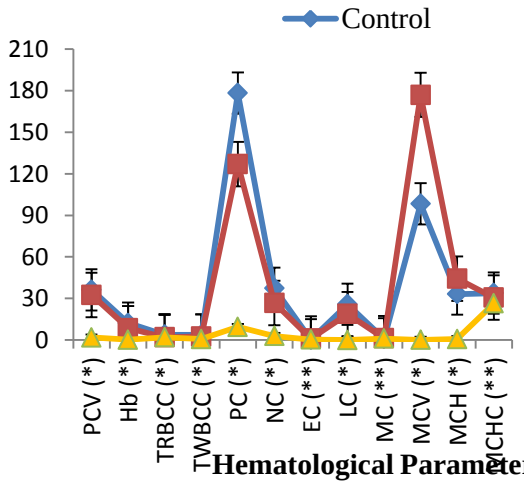
RESULTS

Figure I: Showing Changes in Haematological variables for Malaria Infested, Untreated Mice



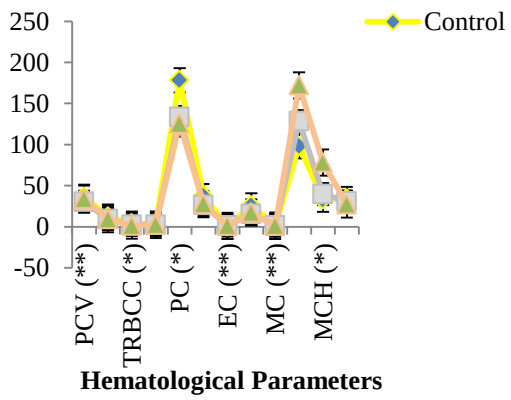
*Grp 1: fed standard diet with no induced malaria, Grp 2: Malaria Induced, Untreated. * = significant at p ≤ .05, while **= insignificant at p > .05. Here, a statistically significant difference was observed for all but MCV, MCH and MCHC upon comparison (PCV, Hb, TRBCC, TWBCC, PC, NC, EC, LC, and MC) between groups.*

Figure II: Haematological Changes in Malaria Infested, Vitamin C. Administered Mice



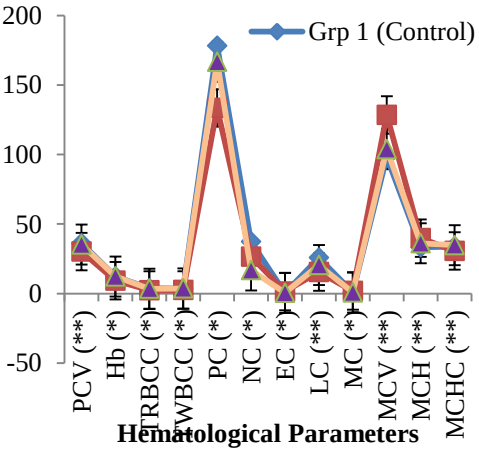
** = significant at p ≤ .05, while **= insignificant at p > .05. From above figure, Result showed no statistically significant difference in EC, MC, and MCHC. However, a statistically significant difference was observed for PCV, Hb, TRBCC, TWBCC, PC, NC, LC, MCV, and MCH upon comparison.*

Figure III: Haematological Changes in Malaria Infested, Vitamin E. Administered Mice



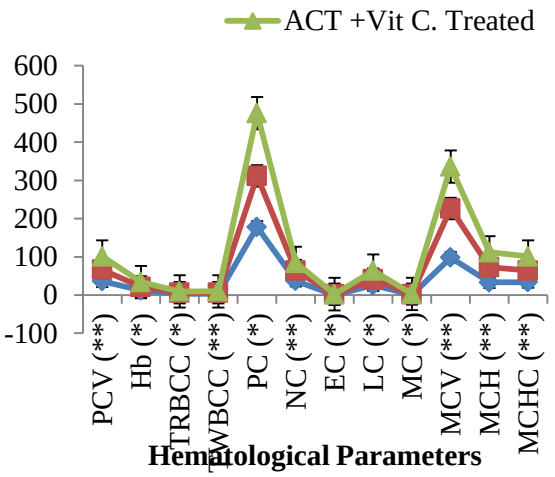
** = significant at p ≤ .05, while **= insignificant at p > .05. Comparison of Hematological parameters for mice of above groups (fig. IV) showed a statistically insignificant difference for PCV, EC, MC, and MCHC, with a statistical significance observed in Hb, TRBCC, TWBCC, PC, NC, LC, MCV, and MCH.*

Figure IV: Haematological Changes in Malaria Infested, ACT Treated Mice



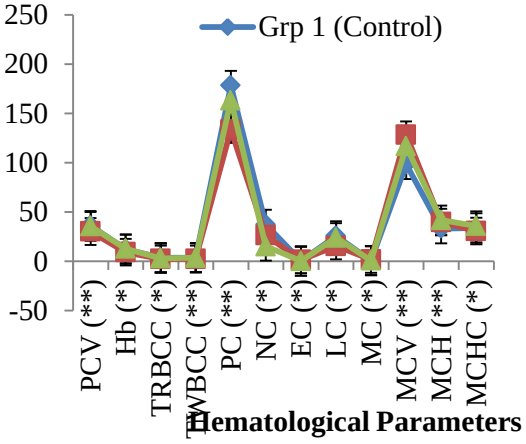
** = significant at p ≤ .05, while **= insignificant at p > .05. Comparison of haematological parameters for mice of above groups (fig. V) revealed a statistically significant difference for Hb, TRBCC, PC, NC, EC, and MC; whereas; PCV, LC, MCV, MCH, and MCHC returned a statistically insignificant difference between groups*

Figure V: Haematological Changes in ACT and Vitamin C Co-administration to Malaria Infested



** = significant at p ≤ .05, while **= insignificant at p > .05. Comparison of Hematological parameters for mice of above groups (fig. VI) returned a statistically insignificant difference for PCV, TWBCC, NC, MCV, MCH, and MCHC; However; Hb, TRBCC, PC, EC, LC and MC were statistically significantly different between groups.*

Figure VI: Haematological Changes in ACT and Vitamin E Co-administration to Malaria



** = significant at p ≤ .05, while **= insignificant at p > .05. From above figure, a statistically significant difference was seen in Hb, TRBCC, NC, EC, LC, MC and MCHC. However, PCV, TWBCC, PC, MCV, and MCH proved insignificant upon comparison between groups.*

DISCUSSION

Hematology is the scientific study of the numbers and morphology of the cellular elements of blood; which consists of the red cells (erythrocytes), the white cells (leucocytes), and the platelets (thrombocytes). In practical terms, the use of these results is vital in the diagnosis and monitoring of disease[15] Hematological studies are of ecological and physiological interest in helping to understand the relationship of blood characteristics to the environment[16] and so could be useful in the selection of humans that are genetically resistant to certain diseases and environmental conditions[17] Haematological parameters are good indicators of the physiological status of animals, [18] and are related to the blood and blood forming organs. [19] While blood act as a pathological reflector of the status of exposed humans to toxicant and other conditions, animals with good blood composition are likely to show good performance and/or resistance to diseases like malaria. [18] Overtime, Malaria infection has been associated with hematological changes and involves cells such as the red blood cells, leukocytes and thrombocytes. [19] With conflicting reports on the effects of malaria on hematological parameters, this study was therefore devised to examine the effect of Vitamins C and E Co-Administration with Artemether Lumefantrene on hematological variables in malaria infected mice

Observed Effects on PCV

Results from present study (Figure I) show that compared with mice of the control group, mean packed cell volume (PCV) was significantly decreased in malaria infested, negative control mice (x = 30.25) who were left untreated than non-inoculated (control) mice (x = 36.25). This implies that on the average, malaria lowered PCV values of mice that were exposed to it. This observation is consistent with previous findings of Marcus *et al.* (2003) who reported malaria infected human subjects to have significantly lower Hb level than non-malaria sufferers, which ultimately results in low PCV. This is also observed to be consistent with previous studies of Iwuji *et al.*, 2012, who reported that co-administration of A/L with Vitamin-E has greater effect in increasing PCV towards normalcy; an effect which may be attributed to the antioxidant capacity activity of Vitamin-E and the A/L as observed by Onyeka *et al.* (2012).

It should be stressed that reports of Agbor (2001) that RBC counts are significantly reduced in malarial infection is consistently in agreement with findings from this present study (Figure 1). Again, Vitamin-C and E administration in malarial infection (Figure 5-7) showed a statistically decreased significant effect on PCV level, with no obvious increase in RBC count. However, administration of Artemisinin / Lumefantrin – (A/L) (figure VII) caused a restoration in RBC count towards normal. This corroborate with findings of previous studies that A/L increases Hb and PCV values with subsequent increase in RBC count. [20] This stimulatory effect of A/L on Hb concentration and PCV levels may be beneficial in antimalarial treatment as malaria causes anaemia.[21] Another study by Agbor, (2001) reports that Artemisinin derivatives caused an insignificant effects on RBC counts, hemoglobin and hematocrit (PCV).

Observed Effects on Haemoglobin Level

Previous studies have reported a significant decrease hemoglobin (Hb) level for malarial infection in comparison to non-malaria infected patients.[21] These findings further corroborates with those of present study for each group of mice (Figure I-X) experimented upon. Contradicting reports from previous studies have also shown that, even though dexamethasone causes an increase in Hemoglobin level by possibly retarding erythrophagocytosis, it however increases erythropoitien production in the kidney. [22] Administration of A/L restored the Hb level towards normalcy; making it consistent with previous studies that showed Hb increased level following recovery from acute

infections.[23, 24] This effect of increasing Hb level by A/L is higher in mice co-administered with Vitamin-C and E; proving that Vitamin-C and E may help the body in its quest to increasing the Hb level.

Observed Effects of Malaria on Total White Blood Cell Count (TWBCC)

As part of its research objectives, current study also investigated the changes in TWBCC variables in immunocompromised mice treated with various combinations of ACTs and Oxidative vitamins. Theoretically, White blood cells (WBCs) are the known to be the centre of target mostly by malarial infection. Ani *et al.*, (2016) had reported a significantly higher value of total WBC (leucocyte) count in malaria positive individuals as against non-malaria infected subjects. Their reports contradict findings of this study and those of Onyeaka (2012) which showed a statistically significant decrease in mean values of total leukocyte count of malaria positive individuals. However, earlier report by Adeleye *et al.* (2012) shows that Coartem can increase total WBC counts, which they attributed to immunological response induced by the drug at variance with the observation.

Importance of Study

Study will provide relevant insight and explanation on the effects of Vitamin C and E co-administration with Artemisinin-based combination therapy (ACT) on haematological parameters. It will also provide useful means of boosting immune response of malarial infected individuals receiving ACT.

Conclusion and Recommendations

Malaria infection is known to cause changes in hematological parameters. In this study, parasite density was seen to be reduced after the intake of Artemisinin / Lumefantrin (A/L). Treatment of malaria with A/L improved and restored deranged hematological parameters, tending it towards normal. Co-administration of A/L with vitamin-C and E had a significant potential to cause recovery from malarial infection; however, it only had effect on certain hematological parameters in this study. Therefore, with Anaemia being a major haematological disorder in malaria infected patients, the use of A/L therapy would lead to greater clinical and haematological benefits, following the recovery period of malaria infection. It is therefore highly recommended.

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