ASTEMIZOLE-METHYLENE BLUE COMBINATION THERAPY REDUCES MONOTHERAPY ADVERSE EFFECTS IN BALB/C MICE

ABSTRACT

Aims: To determine the toxicity of astemizole-methylene blue combination therapy as effective candidates for therapeutic repurposing against malaria.
Study design: Randomized block study design
Place and Duration of Study: Department of Tropical and Infectious Diseases, Institute of Primate Research, between July and December, 2019.
Methodology: The Lorke's technique was used to evaluate the toxicity of the drug combinations in Balb/c mice (N=25). The mice were monitored for clinical signs at 2-hour intervals. After 48 hours, the mice were euthanized, and their tissues collected, weighed and grossly examined. Biochemistry and hematological tests were performed after blood samples were collected. Analysis of Variance and the t-test were used for statistical analysis; differences were considered significant if P values were less than 0.05 ($p< 0.05$).
Results: The findings revealed that mice treated with methylene blue alone experienced a decrease of appetite, while mice treated with astemizole alone experienced slight tremors, which were not observed in the medication combined groups. When compared to the negative controls, the astemizole-methylene blue 3:1 combination group exhibited reduced heart ($p=0.007$) and liver ($p=0.0001$) mean weights. Platelet levels in the astemizole-methylene blue 3:1 group were lower in comparison to the other groups ($p=0.005$), according to the hematological data collected.
Conclusion: When delivered in ratios with less astamizole, astemizole-methylene blue combination therapy showed better results in terms of safety than monotherapy with either drug alone.

Keywords: Astemizole, methylene blue, drug repurposing, toxicity, combination therapy

1. INTRODUCTION

Toxicity testing is an important step in the drug development process for it informs clinicians and researchers on the safety profile of the chemotherapeutic interventions they are using on patients in clinical trials [1]. Traditional approaches from drug discovery to clinical candidate development are costly and time-consuming (over 5 years in most situations) [2]. Furthermore, a lack of sufficient funding tends to further prolong the process of extensive testing and evaluation [3]. Therefore, drug repurposing or repositioning, a strategy that identifies novel therapeutic uses for currently available medication and drug candidates, offers a less expensive and a faster alternative to generating new treatments, including malaria treatments [4].

Although astemizole (AST) was pulled from the market owing to its tendency to produce cardiac arrhythmia when delivered in large doses due to the blockage of the hERG potassium channel, new research has shown that it can offer antiplasmodial action against *falciparum* malaria [9, 10]. At the H_1-receptor sites in the gastrointestinal tract and bronchial muscles, astemizole competes with histamine

[7]. It acts as an anti-plasmodial drug against *falciparum* malaria by preventing the crystallization of heme, a by-product of hemoglobin breakdown that occurs during the *Plasmodium* life cycle's intra-ethryrocytic stage [8]. Following the accumulation of the by-product, parasite death occurs.

On the other hand, methylene blue (MB) is a phenothiazinium salt that was originally used as a textile dye [9]. It has been repositioned and used in malaria treatment for years [12,13]. It works by blocking *Plasmodium* glutathione reductase, an enzyme required for cell development and heme polymerisation in malaria parasites. Heme is a harzadous by-product of hemoglobin breakdown [13,14].

According to Nyirongo *et al.,* [10], MB and AST have demonstrated good antimalarial potential as monotherapies, but their combination *in vivo* is still unknown. Repurposing and combination therapy are two of the tactics being used to produce newer antimalarials in response to the rapidly developing *Plasmodium* resistance [14]. However, the antimalarial candidates resulting from the fusion of repurposing and combinational therapies must be examined for safety. Following the recent investigation on the effects of AST-MB combination against *P. falciparum in vitro* [10], this study demonstrates the toxicological consequences of AST-MB combination in a mouse model.

2. MATERIALS AND METHODS

2.1 Study site

The study was conducted at the Tropical and Infectious Diseases Department (TID), Institute of Primate Research (IPR), Karen, Nairobi County, Kenya.

2.2 Preparation of pharmaceutical solutions

Stock solutions of 1 mg/ml anhydrous methylene blue (Sigma, Germany) and astemizole (sourced from University of Cape Town's Department of Chemistry) were prepared as previously described by Nyirongo *et al.*; Mwangi *et al.,* [10,14]. Similarly, astemizole-methylene blue drug combinations in ratios of 1:3 and 3:1 were prepared and stored at 4^0C until needed [12, 17].

2.3 Experimental animals

Six week old healthy Balb/c mice (15 males and 10 females) were randomly assigned to 5 groups of 5 mice, each weighing 20 ± 2 g. The mice were housed in typical Makrolon type II cages with clear labels as per following groups: Astemizole alone (AST), Methylene blue alone (MB), AST-MB 1:3, AST-MB 3:1 and a negative control with normal saline. Water and food were provided *ad libitum*. The room temperature was maintained at 22°C with relative humidity of 60%-70%.

2.4 Toxicity assessment

For 48 hours, toxicity was assessed using a modified version of Lorke's acute toxicity technique. Healthy Balb/c were given 10 mg/kg doses of MB alone, AST alone, and test drug combinations [19,

20]. The test medicines were given intraperitoneally to five groups: astemizole-methylene blue at a 1:3 and 3:1 combination ratio (AST-MB 1:3 and AST-MB 3:1), methylene blue (MB alone), astemizole (AST alone), and a fifth group that received saline acting as a negative control.

Clinical symptoms, behavioral patterns, and physical parameters (animal body weight, amount of food and water consumed, the coloration of fur, eyes, ears, skin, and tail) were observed and recorded every 2 hours for 48 hours.

The mice were euthanized with carbon dioxide gas at the end of the 48 hours. For hematology analysis, whole blood was collected through cardiac puncture into Ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. For, biochemical analysis, whole blood was collected in 2 ml Eppendorf tubes and left to stand overnight prior to serum separation . The serum was stored at -20^{0}C until it was needed for analysis. Before being stored in 10% buffered formalin, the heart, lungs, spleen, liver, kidneys, and brain were extracted and examined for gross abnormalities. The mean white blood cell count, red blood cell count, platelet count, mean corpuscular hemoglobin, hematocrit count, and hemoglobin concentration were all included in the hematological analysis. To determine liver functionality, biochemical tests included including aspartate aminotransferase, alanine aminotransferase and total protein assays were conducted.

2.5 Data analysis

The t-test was used to compare statistical differences means between controls and treatment groups, while ANOVA was used to compare differences between and within groups, with a Tukey Post Hoc test conducted when there was significant difference after the ANOVA test (SPSS 20). Statistical significance was considered at p-values less than 0.05 ($p< 0.05$).

3. RESULTS

3.1 Clinical signs and symptoms

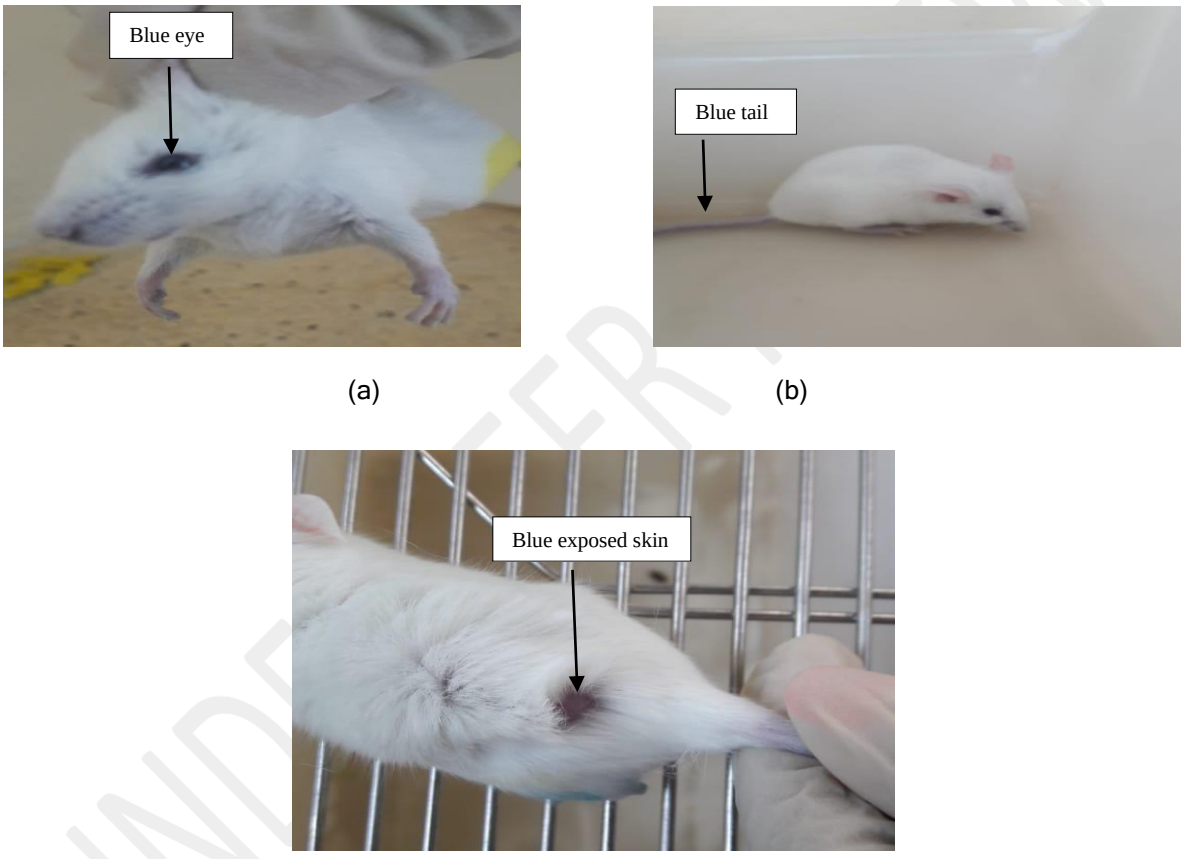
Astemizole, methylene blue, astemizole-methylene blue drug combinations in various ratios did not have any significant effects on the mice body weights, and water consumption between 0-24 hours and 24-48 hours post drug administration. However, a decrease in appetite was observed in the MB alone group between 0-24 hours.

The treatments administered, appeared to affect the behaviour in the Balb/c mice from all treated groups. In the initial 0-24 hours post-treatment, mice in all the treated groups were observed to cluster at one corner of the cage suggesting lethargy. Further to this, it was observed that mice in the AST alone treated group had minor tremors suggestive of neural interference. However, at 24 hours post-treatment to the end of the study period, the animals were active and exhibited normal behaviour.

Changes in urine colour and pH were also observed. Between 0-24 hours, urine colour in the MB alone group was blue and this changed to blue-green between 24-48 hours post drug administration. The urine colour in the drug combination groups was blue-green between 0-24 hours and changed to green between 24-48 hours post treatment. However, the urine colour in the AST treated group, was normal

(umber) throughout the duration of the study. Despite the colour differences, the pH of the urine in all the groups ranged between 5 to 8. Furthermore, the eyes, ears, skin, tails and mouths in the mice treated with MB alone and the AST-MB combination groups had blue colouration within the first 24 hours post-treatment (Fig 1). The eyes, ears, skin, and tails in these groups regained their normal colour between 24-48 hours post drug administration. No colour changes were detected in the same organs in the AST alone treated groups in the same period. The blue colouration that was observed is attributed to MB's characteristic colour. Despite this, the appearance and texture of the fur remained normal in all the groups throughout the 48 hours. Fecal pellets were normal and formed in all the mice, except in 2 mice in the AST-MB 3:1 group that had loose stool with mucus at 26 hours' post-treatment.

Fig 1: Photograph of Balb/c mice 0-24 hours post-treatment



(a) (b)

(c)

(a) blue eyes, (b) blue tail and (c) exposed skin as observed in the mice in the MB alone and AST-MB combination groups.

3.2 Clinical biochemistry

Biochemical analysis which included alanine aminotransferase, aspartate aminotransferase and total protein were done to examine the functionality of the liver, kidney, and heart in mice post-treatment.

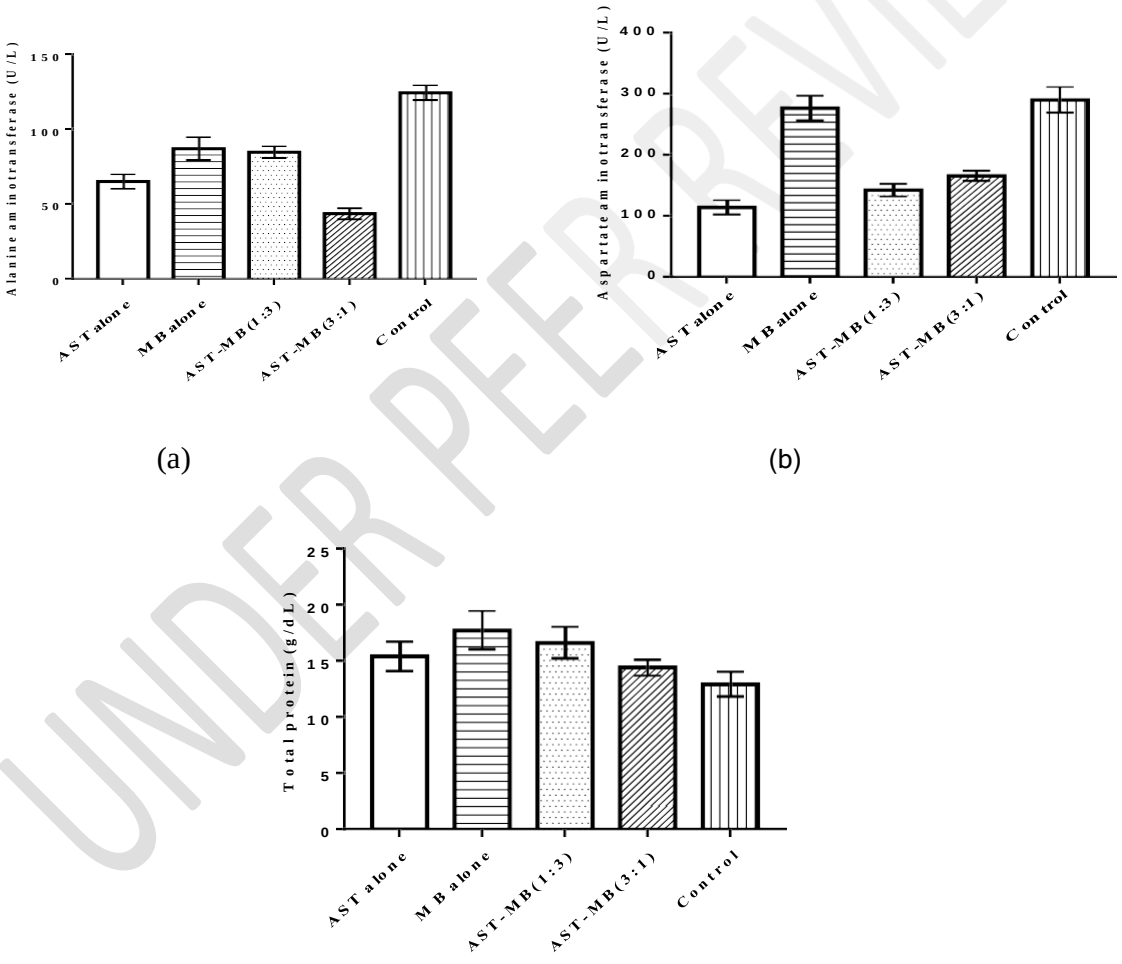
Levels of serum alanine aminotransferase in mice from all the treated groups (67.6 U/L for AST, 86.8 U/L for MB, 84.5 U/L for AST-MB 1:3 and 43.4 U/L for AST-MB 3:1) were lower than in the control group

(124.2 U/L) (Fig 2a). Significantly, there was a difference between the AST-MB 3:1 and control groups (p=0.046).

The results also showed that aspartate aminotransferase levels of mice in the treated groups were lower (113.9 U/L for AST, 276.4 U/L for MB, 142.0 U/L for AST-MB 1:3 and 165.6 U/L for AST-MB 3:1) as compared to the negative control group (289.8 U/L) (Fig 2b).The differences in the aspartate aminotransferase enzyme levels were not significant (p = 0.273).

The total serum protein levels in all the treatment groups were higher than in the negative control group (15.4 g/dL for AST, 17.7 g/dL for MB, 16.6 g/dL for AST-MB 1:3 and 14.4 g/dL for AST-MB 3:1) and 12.9 g/dL in the control groups (Fig 3c). However, the differences were insignificant (p= 0.878).

Fig 2: Biochemistry analysis of Balb/c mice treated and control groups after 48 hours



(a) (b)

(c)

(a) Mean alanine aminotransferase levels (U/L); (b) Mean aspartate aminotransferase levels (U/L); (c) Mean total protein levels (g/dL)

(b)

3.3 Haematological analysis

To determine the effect of the test drugs and combination rations used on blood cells, hematological tests were done 48 hours post-treatment. Overall low white blood cell counts (WBC), mean corpuscular hemoglobin (MCH) and platelet count (PLT) were observed in the treated groups compared to the negative control.

Fig 3a illustrates the WBC count in the animals. Generally, all the treated groups displayed low mean count relative to the untreated negative controls (3.2 x 10^3/µl for AST, 3.4 x 10^3/µl for MB, 4.9 x 10^3/µl for AST-MB 1:3 and 4.9 x 10^3/µl for AST-MB 3:1 in comparison to the 5.5 x 10^3/µl for the negative control group). Of the four treatments, AST-MB 1:3 and AST-MB 3:1 groups had the highest WBC counts (4.9 x 10^3/µl) while the AST alone group was the lowest (3.2x10^3/µl). The difference in the WBC counts of the treated groups compared to the controls was not statistically significant (p=0.600).

The mean PLT count of the mice from all the treated groups was lower (569 x 10^3/µl for MB, 906 x 10^3/µl for AST-MB 1:3 and undetectable for AST-MB 3:1) in comparison to that of the control group (1099 x 10^3/µl). In the AST alone group, the PLT count was higher (1517 x 10^3/µl) than that in the control group (Fig 3b). Despite this, significant differences were only notable in the AST-MB 3:1 treated group (p=0.005), suggesting toxicity of the ratio combination in Balb/c mice.

Similarly, low MCH levels were detected in AST only, AST-MB 1:3 and AST-MB 3:1 treated groups (16.95 pg, 23.86 pg and 17.21 pg, respectively) compared to the control (25.82 pg), only the MB alone treated group had the highest MCH levels (31.61 pg) (Fig 3c). Among the treatment groups, AST-MB 3:1 group had the lowest mean corpuscular hemoglobin (16.95 pg). However, these differences were not statistically significant (p=0.083).

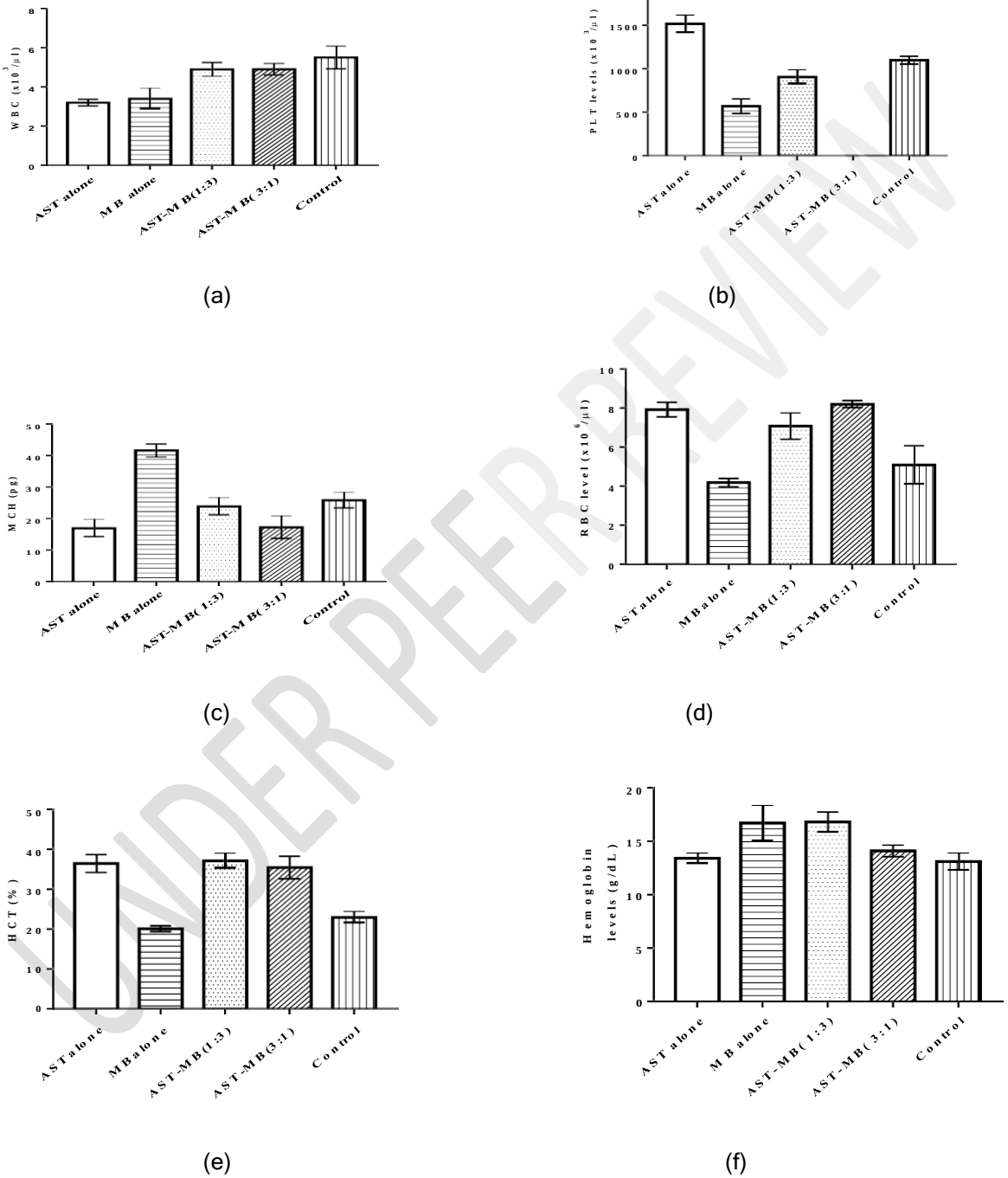
It was also observed that the RBC counts, HB levels and hematocrit levels had increased following treatment as compared to the untreated negative control group. The results showed a slightly elevated RBC count in all treated groups (7.92 x 10^6/µl for AST, 7.07 x 10^6/µl for AST-MB 1:3 and 8.2 x 10^6/µl for AST-MB 3:1) except the MB alone group (4.18 x 10^6/µl) when compared to the control group (5.09 x 10^6/µl) (Fig 3d). Among the treated groups, the AST-MB 3:1 treated group had the highest RBC count while the least was observed in the MB alone group. These differences were however not statistically significant (p=0.168).

Hematocrit levels in the treatment groups were higher in AST, AST-MB 1:3 and AST-MB 3:1 (36. 56%, 31.13% and 35.42% respectively) than those in the control group (22.95%). Interestingly, the MB only treated group of mice had the lowest haematocrit (20.10%), lower than even the controls (Fig 3e). However, differences between the treatment groups and control groups were statistically insignificant (p=0.345).

In another hematological parameter evaluated it was revealed that HB concentration in all the treatment groups was higher (13.4 g/dL for AST, 16.7 g/dL for MB, 16.8 g/dL for AST-MB 1:3 and 14.1 g/dL for

AST-MB 3:1) than that in the control group (13.1 g/dL) (Fig 3f). Overall, there were no significant differences in the HB levels (p=0.440).

Fig 3: Hematological parameters of Balb/c mice, 48 hours after treatment with AST, MB, and AST-MB combinations



(a) Mean white blood cell count (x 10^3/µl); (b) Mean platelet count (x 10^3/µl); (c) Mean corpuscular hemoglobin (pg), (d) Mean red blood cell count (x 10^6/µl) ;(e) Mean hematocrit (%) and (f) Mean hemoglobin concentration (g/dL)

3.4 Gross pathological examination of Balb/c harvested organs

The organs (the heart, liver, kidneys, spleen, lungs, and brain) were harvested. Gross pathology was done by the macroscopic observation of the sacrificed animals and their organs. This provided a general overview of the drug's effects on the organs. The colour, morphology, and weight of the organs were observed and recorded.

On dissection, it was observed that in all MB containing treatments there was blue staining on the animal skin, concentrated at the injection site (Fig 4). The mean organ weights varied between the treatment and control groups after the 48 hours (Table 1).

Except for the mean weights of spleens of the AST alone treated mice (p=0.999), lungs of the AST alone and AST-MB 3:1 treated mice (p=0.999 and p=1.000 respectively), and brains of the mice treated only with AST (p=0.826); all the harvested organs from the treated groups weighed less than those of the control group. These differences were not statistically significant.

The hearts and livers of the AST-MB 3:1 treated group (0.100 ± 0.008 g and 1.002 ± 0.075 g, respectively) weighed significantly less than those of the other groups, particularly the negative control (p=0.007, p=0.001 respectively).

Fig 4: Photograph of sacrificed Balb/c mouse showing the blue discoloration of skin at the point of drug administration

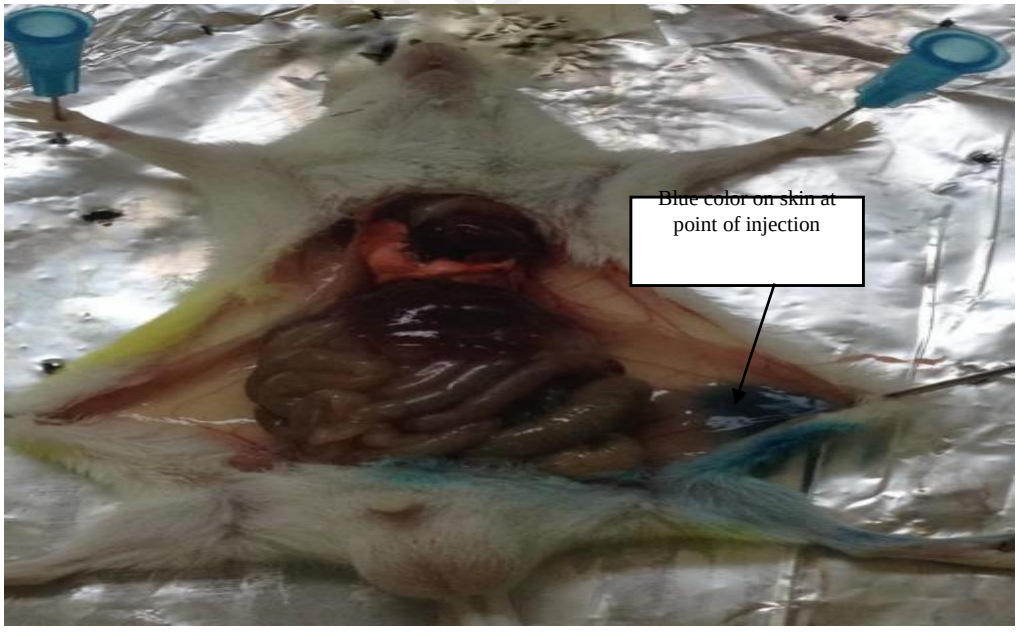


Table 1: Weights (Mean ± SEM g) of harvested organs from treated mice

Organ	*Mean Weights (g) ± SEM; n=5*					
	MB alone	AST alone	AST-MB (1:3)	AST-MB (3:1)	Control	P-value
Heart	0.128±0.008	0.132±0.008	0.141±0.008	**0.100±0.008***	0.136±0.008	0.007
Liver	1.144±0.075	1.114±0.075	1.217±0.075	**1.002±0.075***	1.296±0.075	0.001
Kidney	0.344±0.034	0.358±0.034	0.343±0.034	0.372±0.034	0.372±0.034	0.854
Spleen	0.723±0.016	0.099±0.016	0.090±0.016	0.106±0.016	0.095±0.016	0.298
Lung	0.154±0.016	0.179±0.016	0.166±0.016	0.170±0.016	0.167±0.016	0.326
Brain	0.376±0.037	0.458±0.037	0.397±0.037	0.404±0.037	0.419±0.037	0.279

Statistically significant * (ANOVA, df=4)

4. DISCUSSION

The tremors observed in the astemizole alone treated group were consistent with observations by Riordan *et al* [19], in which tremors were observed in mice administered with astemizole. Astemizole is known to cause long QT syndrome [20] and arrythmias is one of the symptoms of this syndrome [21]. Between 24-28 hours post-treatment, all the mice from the various experimental groups started exhibiting normal behaviour, indicating that a significant portion of the treatment had been metabolized, excreted and the effects wore off. The change in social behaviour was consistent with MB and AST half-lives (5-6 hours and 24 hours, respectively).

Interestingly, despite the blue colouration in the urine in all mice that received any form of MB regimen, only 2 mice from the AST-MB 1:3 excreted formed blue-stained fecal pellets at 26 hours post-treatment. The discolouration in the skin, snout, and tail (on the MB alone and AST-MB combination groups) and blue tinge colouration in the urine and fecal droppings of the mice in these groups were similar to observations reported by Prakash *et al* [22]. The discolouration was self-limiting and harmless [23]. The AST in the combinational experimental group may have played a role in the hydrogenation of MB to a reduced form, leucomethylene blue, that is colourless. Although the urine was not colourless, the greenish-blue colouration was indicative of reduced MB compared to the intense blue (oxidized MB) in the MB-alone treated mice. The mild blue urine colour intensity was a result of an increased biological redox reactions during MB metabolism in the presence of the AST that has Reactive Oxygen Species (ROS)-protective effects [6]. As illustrated in previous studies, while in the presence of glucose, methylene blue is colourless (reduced form) and becomes blue in its oxidized form [24]. Further, astemizole acts as an anti-oxidant [25], meaning that astemizole would favour the formation of the colourless form of methylene blue thus the different hues of the urine in the two AST-MB groups. However, achieving the colourless form is highly dependent on the concentration. Here, however, only

a reduction in color intensity was observed and no further biochemical analalysis were pursued. In both treatment and control groups, there was 100% survivorship over the 48 hour observation period.

In biochemistry, the aminotransferase is an essential enzyme that is part of the normal cellular metabolism processes, particularly the hepatocytes [26]. Alanine aminotransferase catalyses the amino acids to produce oxaloacetate which aids in energy generation. It is mostly found in the liver but considerable concentrations can be found in the kidneys, heart and skeletal muscles [27]. In medicine, the presence of elevated transaminases in serum is a biomarker of liver integrity or hepatocellular damage and an important intermediary enzyme in several metabolisms.

Aspartate aminotransferase, an enzyme that aids in gluconeogenesis and amino acid metabolism by catalysing the transfer of amino groups, was normal in all groups [28]. It is predominately found in the heart and liver.

Total protein is a measure of the amount of albumin and globulin and is a biomarker of liver or kidney anomalies [32, 33]. These results suggested that mice in all the groups had normal total protein levels, thus normal amounts of albumin and globin despite the treatments.

As per levels of alanine aminotransferase, aspartate aminotransferase and total protein, all test drugs except AST-MB 3:1, had no negative biochemical interruptions in the animals. The reduced levels of alanine aminotransferase in the mice following AST-MB 3:1 treatment suggested that this combination was injurious to the liver.

Generally, the test drugs had no significant effect on the haematological profile except for platelet volume, where it dropped to below detectable levels in the AST-MB 3:1 treated group. This suggested that AST-MB 3:1, induced thrombocytopenia in the mice. This observation concurs with findings by Visetin and Liu [31] who observed and attributed very low platelet counts to drugs administered. Further to this, antihistamines such as astemizole have been known to interfere with the structural components of plasma [32].

The AST-MB 3:1 treatment was associated with lower mean heart and liver weights of the mice. The low weight of the liver could occur as a result of toxicological changes within the organ [33]. This low liver weight in the AST-MB 3:1 group is consistent with low ALT levels within the same group, suggestive that the ratio of combination used was detrimental to the organ. Interestingly the AST levels from the same group were much higher, concurring with observations by Kim *et al.*[27] where aspartate aminotransferase levels higher than alanine aminotransferase were attributed to liver abnormalities. Astemizole causes cardiac problems as previously observed in a study by Lee *et al.* [33]. These results, therefore, demonstrate that astemizole in the AST-MB 3:1 drug combination was the main cause of the heart and liver anomalies observed in this study. It is possible that if the study period lasted longer, organ congestion or failure will have occurred. In summary, these results suggested that a 3:1 AST-MB combination ratio was relatively toxic *in vivo*, particularly affecting the platelets, heart and liver function.

5. CONCLUSION

In this study, acute toxicity tests showed that astemizole alone, methylene blue alone and astemizole-methylene blue 3:1 and 1:3 did not cause any mortality of the Balb/c mice. Methylene blue alone treatment affected appetite while the astemizole alone treatment induced minor neurological disturbances (tremors) in Balb/c mice. Reduced appetite and tremors were not observed in the drug combination groups. Astemizole-methylene 3:1 drug combination had a negative impact on the platelet count and caused biochemical and weight changes in the mice liver and heart. However, astemizole-methylene blue 1:3 combination had a better outcome than the monotherapies. The results in this study infer that a high AST dosed AST-MB combination therapy has hematalogical, biochemical and organ damaging potential. Thus, administering a low AST dosed AST-MB drug combination (with less astemizole in the ratios) was safer. This study was limited by time to fully appreciate the long term effects of the dose and combination ratios tested. We recommend that more studies be conducted to investigate the safety and tolerance of the tested combination ratios, dose and drugs in the long term.

Ethical Approval

The tests were carried out in compliance with the Animal Care and Use Committee (ACUC) of the Institute of Primate Research and using study protocols appoved by the Institutional Scientific Ethics Review Committee (Study Clearance Number ISERC/09/2017).

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