Haematological Profile of Male Rats Treated with Ethanol and/or Chloroquine and fed Normal or Low Protein Diet

E Mbajiorgu, T Aire, W Volk, M Albert, L Debusho

Citation

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Abstract

Chloroquine (Q) is widely used as the main drug for malaria therapy in malaria endemic regions of the world, especially in Africa. Individuals on Q therapy frequently consume alcohol (E), in the face of protein malnutrition. This study was designed to evaluate the effects of chloroquine and/or ethanol treatment on haematological values of male Sprague-Dawley rats placed on normal protein (NP, 15%) or on low protein (LP, 6%) diet (malnutrition) for 40 days divided into 4 equal intervals. After 10 days of treatment, 5 - 7 rats were randomly selected from each rat cohort and sacrificed. This was repeated for 4 consecutive intervals. Haematological values were lower in LP than in NP-treated rats during all intervals. Ethanol or Q treatments generally depressed haematopoiesis and produced significant reductions in the values of some of the haematological parameters, viz Rbc, Hb, PCV, MCV, MCH and platelets, irrespective of the dietary protein status. However, the reduction in these parameters due to Q was less in NP-treated rats. Ethanol-low protein treatment (LPE) caused significant elevations (P<0.001) in MCHC values at all intervals except at Day 30. Ethanol and Q interactively enhanced haematopoiesis in NPEQ and LPEQ rat cohorts and caused general and significant increases in the values of some of the haematologic parameters. The greater increases in haematological values of NPEQ than in LPEQ-treated rats, suggests a modulating effect by nutritional status on the effects of these drugs - individually and in combination - on haematopoiesis. A significantly strong interaction (p<0.0001) was found between the drug effects, the sampling intervals and the dietary status.

Total white blood cell count was significantly (p<0.05) increased in the LPE and LPQ-treated rats. The variations in leukocyte counts were essentially caused by variations in neutrophil and lymphocyte numbers. The gradual increases in neutrophil and lymphocyte counts across the intervals in all treated groups reflect a stressful condition impacted upon the immune system. The interaction between the drugs in NPEQ- and LPEQ-rat cohorts resulted in reductions in the lymphocyte and neutrophil counts, suggesting a lowering of immune status of the animals.

INTRODUCTION

Chloroquine is a 4-aminoquinoline drug that is used widely for prophylaxis and treatment of malaria [1,]. Despite the world-wide parasitic resistance to chloroquine [1,], it has remained the drug of choice in the management of plasmodiasis [1,]. In malaria-endemic areas, some individuals on chloroquine treatment or prophylaxis for malaria consume alcohol regularly [1,].

The interaction of chloroquine with some drugs has been shown to either potentiate or attenuate the effects of such drugs [15, 16, 17, 18,]. There is only one report on the interaction of chloroquine with ethanol [1,]. However, concurrent consumption of chloroquine and ethanol in the face of protein malnutrition is prevalent in much of the third world, and therefore may constitute a very serious health problem.

The present communication reports haematological changes that are associated with the concurrent intake of alcohol, chloroquine and normal or low protein diets by adult male Sprague-Dawley rats.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Adult male Sprague-Dawley rats (≥ 3 months in age), body weight 150.5g to 300g, were used in the study. The rats were bred, housed and maintained at the Animal House, Faculty of Medical Sciences, University of Zimbabwe. The rats were housed in a fly-proof accommodation and maintained on a 12-hour light / 12-hour dark regimen. The cages were designed such that the experimental rats were housed in the upper compartment while their faeces and urine pass straight to a lower compartment filled with saw dust thereby
preventing any contamination of the rats. Further Laboratory and data analysis were carried out at the University of Limpopo.

EXPERIMENTAL DESIGN
The rats were randomly assigned to two major groups based on their dietary protein status: Normal protein diet (NP; 14.94%) and low protein diet (LP; 6.19%) groups – see Table 1 for composition and proximate analysis of the feed. Each diet group of rats was further sub-divided into four sub-groups of 20 to 28 rats each that received either vehicles only (ie. normal protein control- NPC; low protein control-LPC), or chloroquine (normal protein chloroquine-NPQ; low protein chloroquine- LPQ), or ethanol (normal protein ethanol-NPE; low protein ethanol-LPE), or both (normal protein ethanol and chloroquine-NPEQ; low protein, ethanol and chloroquine-LPEQ). The rats were on the treatments for either 10, or 20, or 30 or 40 days (d). The intervals were selected in an attempt to monitor the pattern of changes in the treatment effects. Food and water were provided ad libitum and all the animals were placed on the appropriate diet one week before the experiment began.

Chloroquine (10mg/kg body weight per rat) was administered intramuscularly, on days 0, 10, 20, and 30 to the NPQ, LPQ, NPEQ and LPEQ groups while 6% alcohol in drinking water was provided ad libitum to the NPQ, LPEQ and LPEQ groups as described by Nolan et al. [9] and Akingbemi and Aire [10]. Drinking water, being the vehicle for ethanol, was given to the groups on only chloroquine treatment (NPQ; LPQ) while saline, the vehicle for chloroquine, was injected to the groups receiving only ethanol (NPE; LPE). At the interval of ten days (d), after each of the chloroquine injections (10, 20, 30 and 40d), 5 to 7 rats were randomly selected from each of the eight treatment groups, weighed, deeply anaesthetized, and then sacrificed. The blood was collected by cardiac puncture into sterile tubes containing K-EDTA following the method of Akingbemi and Aire [10] and then appropriately labeled to reflect the treatment groups as well as the intervals of the study.

HAEMATOLOGICAL TECHNIQUES
All the blood samples were subjected to complete blood cell count. Leucocyte (WBC) and erythrocyte (RBC) counts were done on an electronic cell counter (Coulter, UK). The haemoglobin (Hb) and haematocrit (PCV) values were determined by the cyanomethaemoglobin and microhaemotocrit methods respectively, with the commercial Zap-o-Globin Reagent (Coulter Electronics, Republic of South Africa [RSA]). All other erythrocyte indices – mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and the mean corpuscular haemoglobin concentration (MCHC) were calculated from the haematological data obtained. The platelet count and differential leucocyte counts were determined as described by Jain [11].

DATA PRESENTATION AND STATISTICAL ANALYSIS
The descriptive statistical analysis was done using SPSS 12.0. The data were further subjected to a 2 x 4 factorial analysis of variance using the statistical software, statistica 6.1. Values are presented as means ± SEM. All the treated groups were compared with the controls (i.e. NPC) at various intervals. The nutritional effect was assessed by comparing the groups with similar treatment but placed on different diets. The interactive analysis involving the treatments, diets and the intervals was also carried out. Student's t-test was used to test for differences in all the comparisons. A value of p < 0.05 was considered significant.

ETHICAL CONSIDERATION:
Ethical clearance for this study was obtained from the Ministry of Agriculture, Zimbabwe, as stipulated in the Scientific Animal Experiment Act, 1963. Additionally, the SPCA staff made frequent inspections of the animals and their experimental room environment. The University of Limpopo ethical committee also approved the study.

RESULTS
All animals were in good bodily condition throughout the experiment. There were no physical or behavioral changes and unplanned deaths recorded throughout the experiment. The haematological values were obtained from the rats treated with E, and/ or Q and fed either NP or LP diet.

INTERACTIVE EFFECTS
The values were generally significantly lower in LP- than in NP-treated rats across the intervals. The interaction of E, and Q with LP and NP diets for 40 days had significant effects (p < 0.001) in almost all the parameters considered except in RBC, eosinophil and monocyte counts (Table 4). Low protein diet exacerbated the effect of this interaction. The increased RBC count in concurrent administration in the NPEQ- and LPEQ- treated rats (Fig. 1) points to increases in
erythropoiesis.

**Figure 1**
Figure 1: Shows the RBC counts of rats treated with ethanol and/or chloroquine and fed normal or low protein diet.

The increase was more in NPEQ-treated rats. Accordingly Hb concentration was significantly increased (p < 0.05) in NPEQ- (significant at 10; 30d), but generally reduced in LPEQ- treated rats (Table 2). Generally, E and Q, interactively significantly elevated (p < 0.01) PCV value in NPEQ- (beneficial drug interaction) but, reduced (p < 0.01) it in LPEQ-treated rats (adverse drug interaction) (Fig. 2).

**Figure 2**
Figure 2: Shows the effects of ethanol and/or chloroquine treatment over time on PCV value of rats fed normal or low protein diet.

The MCV values were generally lower in NPEQ- and LPEQ- treated rats relative to their E or Q treated cohorts, evident at 40d (Fig. 3).

**Figure 3**
Figure 3: Shows the effects of ethanol and/or chloroquine treatment over time on MCV value of rats fed normal or low protein diet.

From 30 to 40d, the MCV values while being gradually increased in NPEQ group, was gradually decreased in LPEQ group (Fig. 3). It also showed strong interaction between intervals, drugs and diets (Table 4).

**Figure 4**
Table 1: Composition of experimental diets in percentages and the proximate analysis

<table>
<thead>
<tr>
<th>Feed Ingredients</th>
<th>Normal protein diet</th>
<th>Low protein diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat and bone meal</td>
<td>- 5.00</td>
<td>- 5.00</td>
</tr>
<tr>
<td>Cotton cake</td>
<td>- 10.00</td>
<td>- 10.00</td>
</tr>
<tr>
<td>Soya oil cake blend</td>
<td>- 8.00</td>
<td>- 8.00</td>
</tr>
<tr>
<td>Crushed yellow maize</td>
<td>- 23.80</td>
<td>- 15.00</td>
</tr>
<tr>
<td>Sugar</td>
<td>- 13.00</td>
<td>- 13.00</td>
</tr>
<tr>
<td>Molasses</td>
<td>- 5.00</td>
<td>- 5.00</td>
</tr>
<tr>
<td>Soya oil</td>
<td>- 3.00</td>
<td>- 3.00</td>
</tr>
<tr>
<td>Oats</td>
<td>- 6.00</td>
<td>- 8.00</td>
</tr>
<tr>
<td>Monocalcium phosphate</td>
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<td>- 0.10</td>
</tr>
<tr>
<td>Salt (coarse)</td>
<td>- 0.50</td>
<td>- 0.50</td>
</tr>
<tr>
<td>NF P1 broiler premix (FG-1520)</td>
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<td>- 0.30</td>
</tr>
<tr>
<td>Maltose residue</td>
<td>- 40.00</td>
<td>- 40.00</td>
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</table>

Proximate analyses of experimental diets

<table>
<thead>
<tr>
<th>Components</th>
<th>Normal protein diet</th>
<th>Low protein diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>79.63</td>
<td>79.63</td>
</tr>
<tr>
<td>Crude protein</td>
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<tr>
<td>Fat</td>
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<tr>
<td>Crude fibre</td>
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<tr>
<td>Ash</td>
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<td>4.99</td>
</tr>
<tr>
<td>Total digestible nutrients (TDN) %</td>
<td>79.63</td>
<td>79.63</td>
</tr>
</tbody>
</table>

Prepared and Analyzed by National Foods (SAFCO), Zimbabwe.
Table 2: The effects of ethanol, chloroquine and both on some haematological indices of rats fed normal or low protein diet

Values are given as mean ± s.e.m. Values in the same row with different superscripts (alphabets) from NPC (controls) are significantly different from NPC.

Values in the row with the same superscripts *(asterisks) are not significantly different from NPC.

Significance levels = a (p < 0.05), b (p < 0.01), c (p < 0.001),
d (p < 0.0001)

Values in the same row with superscript † indicate significant dietary effect on a particular treatment (i.e. NPC/LPC, NPE/LPE etc)
Table 3: The leukocyte count and differential leukocyte analysis of rats treated with ethanol and/or chloroquine and fed low or normal protein diet.

<table>
<thead>
<tr>
<th>Subgroups of rats</th>
<th>NPE in days</th>
<th>LPC in days</th>
<th>NPEQ in days</th>
<th>NPEQ in days</th>
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<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>5.75± 0.64*</td>
<td>6.51± 0.06*</td>
<td>5.70± 0.49*</td>
<td>4.72± 0.45*</td>
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<tr>
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<td>6.90± 0.18*</td>
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<td>7.10± 0.34*</td>
<td>6.60± 0.49*</td>
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<td></td>
<td>6.56± 0.43*</td>
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<td>6.81± 0.49*</td>
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<tr>
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<tr>
<td></td>
<td>1.98± 0.22*</td>
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<td>0.69± 0.03*</td>
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<tr>
<td></td>
<td>0.09± 0.04*</td>
<td>0.07± 0.04*</td>
<td>0.07± 0.04*</td>
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</tr>
<tr>
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<td>0.05± 0.04*</td>
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</tr>
<tr>
<td></td>
<td>0.05± 0.04*</td>
<td>0.11± 0.04*</td>
<td>0.10± 0.03*</td>
<td>0.08± 0.03*</td>
</tr>
</tbody>
</table>

Values are presented as means ± s.e.m. (x10^9). n = no. of animals

Values in the same row with different superscripts (alphabets) from NPC (controls) are significantly different from NPC.

Values in the row with superscripts *(asterisks) are not significantly different from NPC. Significance levels = a (p < 0.05), b (p < 0.01), c (p < 0.001), d (p < 0.0001)

Values in the same row with superscript † indicates significant dietary effect on a particular treatment (i.e. NPC/LPC, NPE/LPE etc)
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Table 4: The level of significance of the analysis of variance on the interaction of ethanol and/or chloroquine on some haematological indices of rats fed low or normal protein diet.

The interaction generally depressed MCH values in NPEQ- and LPEQ- groups relative to other treatment groups but, from 30 to 40d led to elevated values in NPEQ group against a gradual decrease in LPEQ group. While E and Q interactively antagonize each other in LPEQ-treated rats and produce MCHC value that was midway between that of LPE and LPQ at 40d, further reductions was recorded in NPEQ-treated rats at all intervals relative to other groups (Table 2). The strong significant interaction between the drugs, diet and intervals existed despite fluctuations in platelet count across the intervals (Table 4). Slightly elevated platelet values were found in NPEQ- and LPEQ- groups relative to their E and Q cohorts. Leukocytosis caused by E and Q treatments, were interactively reduced in LPEQ- and NPEQ-treated rats (Table 3). Since no significant change was observed in eosinophil and monocyte counts (Table 3), the interaction between interval and treatments, interval and dietary regimens, treatment and dietary regimens and between the three (interval x treatment x diet) was not significant for monocytes and eosinophils, but highly significant for neutrophils and for lymphocytes, except in the interaction between treatment x feed, and interval x feed for lymphocytes only (Table 4).

RBC
The numbers of RBC were significantly decreased (P < 0.05) in the LPC and NPE (10; 20d respectively), LPE (10; 30d), NPQ (20d) and LPQ (10 to 40d) groups relative to their respective interval control (NPC) as shown in Table 2. Low protein diet significantly decreased (P < 0.05) the numbers of RBC in LPC (10; 20d), LPE, LPQ (10; 30d) and LPEQ (10d) groups relative to their respective NP cohort as shown in Table 2.

HB.
The haemoglobin concentrations were significantly decreased (P < 0.05) in the LPC (20; 40d), NPE (10; 20d), LPE (10 to 40d), NPQ (20d), LPQ (10; 20; 30d), NPEQ (20d) and LPEQ (10 to 40d) groups in comparison with the respective interval controls (Table 2). Low protein diet significantly reduced HB concentration in LPC (20; 40d), LPE (10 to 40d), LPQ (10 to 30d), and LPEQ (10 to 40d) groups compared against their NP cohorts (Table 2).

PCV
Normal protein intake probably increased erythropoiesis at 30d and 40d and reversed the significantly depressed PCV value by E and Q treatment (NPE, 10 & 20d; NPQ 20d). The increased erythropoiesis was more in NPE-treated rats hence the significant increases in PCV values (20 and 30d), but normalized for both drugs at 40d (Fig. 2). Low protein intake exacerbated the depressive effects of E and Q in (LPE-) and significantly (p < 0.01) in LPQ-treated rats at 40d. Irrespective of the dietary status, Q seems to have slightly more reductive effect than E. Low protein diet significantly reduced (P < 0.05) PCV in LPC (20; 40d), LPE, LPQ and LPEQ (10 to 40d) groups compared against their NP cohorts (Table 2).

MCV
The MCV values were significantly reduced (p < 0.05) during all the intervals in LPC, LPE, LPQ and LPEQ groups, NPE (10 to 30d), NPQ (10; 20d) and NPEQ (10 to 30d) in comparison with respective interval control (NPC) (Table 2). Significant elevations (p < 0.05) were however recorded in the NPE and NPQ groups at 40d. Low protein diet alone or in combination with ethanol and/or chloroquine significantly (p < 0.05) decreased MCV values during all intervals in comparison with their respective NP cohorts (Table 2).

MCH
In comparison with the respective interval controls, MCH values were significantly (p < 0.05) reduced in LPC, LPE and NPEQ (10 to 30d), NPE (10d), NPQ (10; 20d), LPQ and
LPEQ (10 to 40d). However, LPC and LPE respectively showed significant increases (p < 0.05) in MCH value at 40d (Table 2). Low protein diet significantly reduced MCH value in LPC, LPQ (10 to 40d), LPE (10 to 30d), and LPEQ (10 to 40d) against their NP cohorts (Table 2).

**MCHC**

The MCHC were significantly elevated (p < 0.05) in LPC and LPQ (20; 40d), LPE (10; 20; 40d) and LPEQ (10 to 40d), while significant reductions (p < 0.05) in MCHC were noted in LPC (30d), NPE (40d), NPEQ (10 to 40d) when compared with their respective interval control (NPC) as shown in Table 2. Low protein diet significantly increased MCHC in LPC (20 to 40d), LPE and LPEQ (10 to 40d), LPQ (10; 40d) groups relative to their respective NP cohorts (Table 2).

**PLATELETS**

Platelet counts significantly (p < 0.05) decreased in LPC (40d), NPE and NPQ (20; 40d), LPE (10; 20; 40d), LPQ and NPEQ (40d) in comparison with the respective interval controls (Table 2). Low protein diet significantly decreased (p < 0.05) platelet counts in LPC (40d), LPE and LPEQ (10d respectively), but significant increase in LPE (30d) and LPQ (20d) groups, in comparison with their NP cohorts was observed.

**TOTAL AND DIFFERENTIAL WBC COUNT**

The results of WBC and differential counts are presented in Table 3. The counts were generally slightly higher in the LP-treated rats. However, gradual increases between intervals were observed in NPEQ- and LPEQ-treated rats.

The WBC count was significantly (p < 0.05) increased in LPC (10; 30; 40d), NPE (40d), LPE (10; 20; 40d), LPQ and NPEQ (40d) and LPEQ (10d respectively) and monocyte count in LPC (40d), LPE and LPEQ (10d) respectively, but significant increase in LPC (30d), LPQ (10d to 30) and LPQ (10d) when compared against their paired NP-treated cohorts (Table 3).

**DISCUSSION**

**GENERAL**

The increase in the interactive effect of E and Q by LP-diet is similar to that reported by Akingbemi and Aire [10] on the interaction between gossypol and ethanol in normal and low protein fed-rats. High dietary protein intake increases metabolism of drugs and the activity of the mixed function oxidase system, and consequently increases the excretion of the drugs [13], while inadequate protein intake results in reduced metabolism [13, 14, 15] and therefore increased toxic effects of the drugs. The fluctuations in treatment effects in all the parameters suggest changes in the biotransformation or bioavailability of the drugs across the intervals. The similarity in trend across the intervals in the values of RBC, Hb and PCV agrees with the report of Johnson et al. [15]. The changes produced in RBC indices by E, Q and their interaction under NP- and LP-intake implicates a tendency toward low Hb and small cells. Perhaps microcytic anemia may develop if the treatment continued for a longer duration.

As previously reported by Harold and Ballard [16], the reductions in RBC count in both dietary regimens by E and Q suggests a depression of haematopoietic process. However, both drugs interactively altered their individual adverse effects on the haematopoietic system leading to increased erythropoiesis (Fig. 1). The increase was more amongst the NPEQ-treated rats. Musabayane et al. [16] have reported an interactive effect of E and Q, whereby E and Q in combined administration, significantly increased its excretion. This study has shown that Q may be directly toxic to the haematopoietic system as also reported by Nagaratnam et al. [15]. Since E is also directly toxic to the haematopoietic system [15], the enhancement of haematopoiesis, as the available literature suggests, may be a combination of one or more of the following: (a) alteration of renally active hormones [1], (b) increased metabolism and excretion of both drugs due to high protein intake [12], (c) a metabolic induction (interaction) via cytochrome P-450 enzymes [14] i.e. metabolic or biological antagonism.
The general reduction over time in MCH values in all the treatment groups implicates different levels of anemic conditions. The greater reduction (p<0.01) in LP-fed rats indicates greater severity of effects in low protein intake. The convergent/reductive effects of E and Q in the NP-fed rats (NPE, 30, 40d and NPQ 30, 40d) and the divergent/reductive effects in the LP-fed rats (LPE, 30, 40d and LPQ, 30, 40d) may reveal phases of drug effects and stress on the body’s homeostasis as the treatment progressed. It suggests that interaction between the drugs and other metabolic processes may be involved. The pronounced difference in MCH values of NPEQ- and LPEQ-treated rats at 40d, suggest a modification of drug action, enhanced by the duration of treatment and influence of dietary regimen as already explained, above.

The increased MCHC in LP-treated rats (LPE, LPQ and LPEQ) suggests a hyperchromic anemia, a condition commonly associated with a decrease in number and increase in size and haemoglobin content of RBCs. The general reduction in RBC number may have caused the accumulation of haemoglobin within the available RBCs, while the decreased MCHC values, especially in NPE and NPQ, may be due to the greater number of RBCs in comparison to LPE- and LPEQ-treated rats. The effects of the treatment on other haematologic parameters may have also contributed to the MCHC profile.

At 40d, thrombocytopenia was present in all treatments irrespective of dietary status. Ethanol-induced thrombocytopenia may be due to its interference with the late stage of platelet production [16,32,39], possibly due to a combination of ineffective thrombopoiesis and a shortened platelet life span or both [31]. The increases in circulating platelets in LPE (thrombocytosis) suggest an initial response to toxic effects of E, while the decrease from 30 to 40d may indicate a time-dependent effect with a consequent decrease in haematopoiesis [16]. Low protein-intake exacerbated thrombocytopenia caused by Q as reflected in LPQ treated group at 40d. The present finding conflicts with the report of Osim et al. [37] that Q prolongs the life span of platelets by inhibiting platelet aggregation thereby increasing the number of circulating platelets without any effect on bleeding time [38] but agrees with other reports that Q causes thrombocytopenia [39]. Interactively both drugs acting antagonistically produced less adverse effect in NPEQ- and LPEQ-treated rats.

The higher WBC counts in the LP- than the NP-treated suggests that adequate nutrition is essential in stabilizing the...
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immune system of the body. The leukocytosis (LPE and LPQ), less severe in NPE and absent in NPQ groups, suggests that nutrition modulates the effects of both drugs. The gradual but consistent leukocytosis effect (more in LPEQ than in NPEQ group) due to the interaction between E and Q may indicate a gradual development of a pathological condition, with Q and E consistently ameliorating the effect of each other through the intervals, as suggested by the significant interaction between the drugs, diets and intervals.

Eosinophil and monocyte counts did not contribute to the change in the total WBC count observed in the treatment groups irrespective of the nutritional status, as no significant change in count was observed. Since neutrophils and lymphocytes have a major role in fighting foreign organisms, the variations in leukocyte counts were essentially caused by variations in neutrophil and lymphocyte numbers.

The neutrophilia across the intervals in all the treatment groups reflect stress increases on the immune system. With high level of pathogen fighting cells in the LP-treated rats, the immune system seems to be more challenged in this group, and consequently the significant increase in lymphocyte count at 40d in LPC, 30d in LPE and LPQ-treated rats. The interaction between the drugs in the E-Q-treated rats resulted in reduction in the lymphocyte and neutrophil counts, suggesting a lowering of resistance to infectious agents.

CONCLUSION
There is a strong significant interaction between E, Q, dietary status and duration of treatment on haematologic values. The depressive effects of E and Q, individually, on haematopoiesis is gradual but may become marked with time, even in the absence of protein malnutrition. The individual adverse effects of E, Q, and diet seem to be biologically altered in concurrent administration. Further studies will attempt to determine the probable mechanism of action involved.

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