Original article

Eradication of Transfusion-Induced Malaria by *In vitro* Processing

Mohamed Siddig Mohamed Ali[1], Abdul Gader M. Yousif Kadaru[2]

ABSTRACT

OBJECTIVE: To determine the lethal dose of chloroquine to be added to donors' blood *in vitro* for eradication of transfusion-induced malaria and to study the unfavorable effects of this dose on the constituents of the stored blood.
METHODS: A total of 4484 blood donors, recruited for this study, were screened for malaria parasites microscopically using Giemsa' staining technique. Only 30 blood samples (200ml of blood each) satisfied the inclusion criteria of the study. Each of these blood samples was subdivided equally into four sub-samples to obtain 120 sub-samples. Three different concentrations of chloroquine were added to 90 specimens (30 samples represent each dose) while 30 specimens (control) were left without the drug. Blood specimens were then tested by parasitic, hematological and biochemical techniques on the day of collection and after 24 and 48 hours storage in blood bank refrigerator.
RESULTS: The numbers of malaria parasites killed were proportional to chloroquine doses added to donors' blood. No parasites were killed among the control donors' blood samples. The determined lethal dose of chloroquine was safe to all constituents of the stored blood.
CONCLUSION: For eradication of transfusion induced malaria by *in vitro* processing of donors blood, chloroquine is effective and safe drug. We recommend application of the optimal dose of chloroquine (626.1µg/L) to the components of the blood bags prior to phlebotomy.

Key words: chloroquine, malignant tertian malaria, prothrombin

Transfusion-induced malaria can be a fatal complication of an otherwise simple medical or surgical intervention. It has not been uncommon to find that one infected donor has transmitted malaria to four or more recipients of his blood[1].
Absence of symptoms even for a long period does not ensure lack of infectivity[2].

1.Assistant Professor and Head of the Department of Haematology, Faculty of Medical Laboratory Sciences, AI Neelain University, Khartoum, Sudan
2. Professor of Medicine & Tropical Diseases and Dean, Faculty of Medicine, University of Khartoum, Khartoum, Sudan
For correspondence:
Dr. Mohamed Siddig Mohamed Ali
Faculty of Medical Laboratory Sciences - AI Neelain University. P.O. Box 12702. Khartoum - Sudan
Tel. 0249918031046. **E-mail:** mohdaru@hotmail.com

All known species of malaria are able to survive for days and even weeks in blood stored at 4ºC[3].
Malignant tertian malaria (*P. falciparum*) has been transmitted by blood stored for 14 days[4] while benign quartan malaria (*P. malariae*) has been accidentally transmitted in blood previously stored for 5 days[3, 5].
Consequently large numbers of parasites can be transmitted through this route where as, most patients in need of blood transfusion are already weakened by severe disease. Malaria thus behaves very aggressively in such patients[6].
In Sudan, the infection rate of the malaria parasite among blood donors was 6.5% using direct microscopy (61.9% sensitivity) and 21% using the PCR. This infectivity is

considerable and can lead to a higher risk of complications and fatality in recipients[7].
This risk is worsened by the practical difficulties of the elimination of parasites from donors' blood *in vivo*, before donation[1].
Also, systemic screening of suspected donors is not a practical solution to the problem in malarial endemic countries[1]. Even by using an advanced technique several thousand parasites might be present in one pint of blood and still pass undetected[8].
Moreover, it is neither ethical nor desirable to transfuse infected blood to weak, ill patients, as the induced disease may be difficult to control. A lot of these patients may be receiving many different drugs before transfusion, which may antagonize antimalarial drugs. These facts denote the need for alternative methodologies for the selection of blood to be used in transfusion that could offer fast, cheap and safe results. It is easy, cheap, safe and practical to process donors' blood *in vitro* with low concentrations of chloroquine.
The concentrations given to a patient from a single bag of transfused blood are far low compared to the single dose that might be offered to donors or transfused patients.
Thus, it is essential to determine the dose of chloroquine, lethal to the malarial parasites, to be added to donors' fresh blood and to detect the hematological and biological changes that may occur by adding chloroquine to blood infected with malarial parasites and is stored at 4º – 6º C.

Materials and Methods

This study was conducted in Ahmed Gasim Hospital, Khartoum State, Sudan. Using a 5ml-chloroquine injection which contains 200mg chloroquine base, three serial concentrations of chloroquine phosphate were prepared as follows: 0.5ml (20mg), 1ml (40mg) and 2ml (80mg) of the injection solution were diluted with normal saline to obtain 80ml of each dilution with 250, 500 and 1000 mg/l concentrations consecutively. Next, fifty microliters of each were dispensed into separate 50ml-blood bags to obtain 250, 500 and 1000µg/l concentrations subsequent to the addition of 50 ml of donors' blood.
Sample collection took place between October 2002 and January 2004. All individuals who donated blood in Khartoum state hospitals during the study period were included in the study population if they satisfied the following criteria:
1. Presence of malaria parasite in donors' blood films.
2. Density of infection must range between 1000 and 80000 parasites/µl (only asexual stages were considered).
3. The parasites grow when cultured in the first day.
4. Donors did not receive quinine within the last 7 days, chloroquine within the last 28 days and Fansidar within the last 14 days.
One (200ml) pint (CPDA-1) of donors' malaria parasite infected blood was collected and subdivided equally into four prepared small bags (50ml) using a blood bank mixer (Biomixer-323). Three bags were preloaded with the three concentrations in addition to one bag (control) left without drug. All blood specimens were tested for parasite culture, platelets count, total leukocyte count, packed red cell volume, lysis percentage, osmotic fragility, prothrombin time, activated partial thromboplastin time, sodium and potassium serum levels simultaneously on the day of collection. Thereafter, the bags were stored in the blood bank refrigerator (4º-6ºC) and tested after 24 and 48 hours using the same laboratory procedures.
The microscopic malarial parasite identification technique was performed as described by Cheesbrough M.[9]. The absolute number of parasites (number / µL) was estimated in the thick blood films by counting the parasites against 200 white blood cells, multiplied by the total leukocyte count and divided by 200.
In vitro cultivation of erythrocytic stages of Plasmodium falciparum was intended for the assessment of the sensitivity of fansidar and to confirm the viability or death of the parasites. It was conducted using RPMI-1640 medium (GIBCO™), Invitrogen Corporation. The minimum inhibitory concentration (MIC)

was determined by computerized probit/log dose response analysis, SPSS. Indication of resistance was determined by formation of schizonts at 8 pmol chloroquine per well or more.

For counting platelets and white blood cells, blood samples were diluted 1 in 20 using (1%) ammonium oxalate and 1 in 20 using (2%) glacial acetic acid solutions respectively. They were counted manually by haemocytometry using the improved Neubauer chamber as described by Cheesbrough M.[9].

Partial thromboplastin time is a screening test for the intrinsic clotting system, i.e. factors XII, XI, IX, VIII, X, V, prothrombin and fibrinogen. Prothrombin time is a screening test for the extrinsic clotting system, i.e. factor VII, X, V, prothrombin and fibrinogen. These tests were performed as described by Dacie & Lewis[10] using the reagent of DiaMed company.

The osmotic fragility test gives an indication of the surface area/volume ratio of erythrocytes. It measures the effect of the applied doses on erythrocytes' membrane. It was performed as described by Dacie & Lewis[10].

Packed red cell volume is the proportion of whole blood occupied by red cells which was measured as described by Dacie & Lewis[10].

Percentage of lysis is a real indicator for survival of red blood cells during the storage period. Five mls of each blood sample, just after collection, was centrifuged for 5 minutes at 12000g. The supernatant was stored sterile at 4° C to be used as blank. Five hundred microliters of the precipitated red cells was diluted in 5 mls of pure distilled water and stored at 4° C to be used as standard. Both solutions were used in the determination of percent lysis for the same sample throughout the storage period. After 24 and 48 hours one milliliter of each blood sample was also centrifuged for 5 minutes at 12000g and the supernatant of each was read at 540 nm wavelength. Lysis percentage was calculated by dividing the optical density of the tested supernatant by the optical density of the standard and multiplied by 100.

Serum potassium and serum sodium were measured by the flame photometry using Sherwood Scientific-410 flame photometer. Data were analyzed by computer using SPSS program. Non-parametric tests were mainly used for the abnormal distribution of the data.

Results

The total number of individuals who donated blood was 4956; however, 472 (9.5%) of them were rejected on the following bases: 303 (6.1%) were found to be hepatitis B virus carriers while 169 (3.4%) were found to be HIV positive. The collected blood bags totaled 4484. All were subjected to screening for malaria parasites microscopically using a standard Giemsa' staining technique. A total number of 278 (6.2 %) blood bags' specimens were found to carry malaria parasites. The parasite densities were less than a thousand in 215 (77.3%) while parasite-densities of greater than a thousand were observed in 63 (22.7%) specimens. Of these, 30 (10.8%) samples were found to be resistant to chloroquine. In addition, another three (9.09%) samples showed no growth when cultured on the first day. Thus, the actual number of blood samples for this study was reduced according to the above-mentioned parameters to only 30. All of the accepted blood bags belonged to asymptomatic male donors between 25 and 35 years old.

Table (1) demonstrates the reduction of numbers of malaria parasites when different concentrations of chloroquine were applied to donors' blood stored for forty-eight hours. The control of donors' blood samples (without addition of antimalarials) reveals a stable number of parasites even after 48 hours storage.

Parasite counts decreased with the increasing concentration of chloroquine and elongation of storage period. Thus the parasites disappear after 48 hours in samples with the higher concentration (1000µg/l) of chloroquine.

Table 1

Positivity and mean of the parasite counts during 48-hour storage at 2-8° C

Sample	The day of collection		Twenty-four hours after		Forty-eight hours after	
	Positivity	Mean	Positivity	Mean	Positivity	Mean
C1	30 (100%)	3273	8 (26.7%)	358.67	3 (10%)	47.3
C2	30 (100%)	3273	5 (16.7%)	263.36	3 (10%)	40.00
C3	30 (100%)	3273	4 (13.3%)	65.37	0(0%)	00
Ctrl	30 (100%)	3273	30 (100%)	3273	30(100%)	3273

Key: C1= 250 µg/l chloroquine C2= 500 µg/l chloroquine C3= 1000 µg/l chloroquine
Ctro= control

Table 2

Doses of chloroquine that kill 50%, 95% and 99% of the parasites (respectively) after 24-hour and 48-hour storage in a blood bank refrigerator (2-8 °C)

Storage period	Lethal Doses		
	50% Lethal	95% Lethal	99% Lethal
Twenty-four hours	183.3 µg/l	496.38 µg/l	626.10 µg/l
Forty-eight hours	144.2 µg/l	278.48 µg/l	334.14 µg/l

Table (2) illustrates the lethal doses of chloroquine. It was observed that higher doses were required when the blood was stored for 24 hours versus 48 hours.
Also higher doses of chloroquine were necessary to kill larger numbers of malaria parasites.

Table 3

Prothrombin time and partial thromboplastin time of the stored blood samples

Sample	The first day		24 Hrs Incubation		48 Hrs Incubation	
	PT	APTT	PT	APTT	PT	APTT
C1	15.67	36.20	16.03	37.60	17.60	39.13
C2	15.67	36.20	16.40	38.20	18.3	40.13
C3	15.67	36.20	17.60	39.40	18.97	42.00
Ctrl	15.67	36.20	15.93	38.400	17.97	40.86

Key: C1= 250 µg/l chloroquine C2= 500 µg/l chloroquine C3= 1000 µg/l chloroquine
Ctro= control

Table 3 shows the increase in the mean and standard deviation of prothrombin time and partial thromboplastin time with the increase of chloroquine dose and storage duration. Minimum increase was observed when the minimum concentration of the drug was added.
Although there is significant statistical difference between the result times among different blood samples, still the upper levels are within the normal range.

Table 4

Osmotic fragility values of blood samples during the storage period

Sample	The first day		24 Hrs Storage		48 Hrs Storage	
	Mean	SD	Mean	SD	Mean	SD
C1	0.4510	0.00305	0.4550	0.0153	0.460	0.03051
C2	0.4510	0.00305	0.4650	0.023	0.460	0.02034
C3	0.4510	0.00305	0.4550	0.0153	0.485	0.04577
Ctrl	0.4510	0.00305	0.4600	0.031	0.460	0.03050

Key: C1= 250 µg/l chloroquine C2= 500 µg/l chloroquine C3= 1000 µg/l chloroquine
Ctro= control

Table 4 demonstrates the mean and standard deviation of the osmotic fragilities. It was increased in samples with high doses of chloroquine and with increased storage time. No significant statistical difference was observed between the results of the control and the different blood samples when stored for 24 hours.
When blood samples stored for 48 hours, correlation between processed blood samples and the control reveals statistical similarity, still the upper levels are within the normal.

Table 5
Haematocrit values of blood samples after 24-hour & 48-hour storage at 2-8° C

Sample	The first day		24 Hrs Storage		48 Hrs Storage	
	Mean	SD	Mean	SD	Mean	SD
C1	42.70	2.731	39.20	3.633	37.50	4.125
C2	42.70	2.731	41.07	11.635	39.20	3.428
C3	42.70	2.731	40.40	10.071	37.90	5.013
Ctrl	42.70	2.731	40.50	3.972	39.50	7.960

Key: C1= 250 µg/l chloroquine C2= 500 µg/l chloroquine C3= 1000 µg/l chloroquine
Ctro= control

Table 5 shows that the packed red cell volume was decreased when the time of storage was prolonged regardless of the increase of the doses.
No significant statistical difference was observed between the results of the control and blood samples containing chloroquine during the storage period.

Table 6

White blood cell and platelet counts by storage period

	The first day		24 Hrs Storage		48 Hrs Storage	
Sample	WBC	PLTs	WBC	PLTs	WBC	PLTs
C1	3250	225000	2380	196000	2520	193000
C2	3250	225000	3000	179800	2620	179000
C3	3250	225000	3520	172600	2470	172000
Ctrl	3250	225000	2940	208000	3230	178000

Key: C1= 250 µg/l chloroquine C2= 500 µg/l chloroquine C3= 1000 µg/l chloroquine
Ctro= control. WBC: White blood cell count. PLTs: Platelet count

Table 6 illustrates the mean and standards deviation of the leukocyte and platelet counts. Each parameter was decreased when the storage time was prolonged and did not correlate with the dose concentration.
No significant statistical difference was observed between the results of the control and all of the processed blood samples when they were stored for 24 or 48 hours.

Table 7

The lysis percentage of the stored red blood cells during storage at 2-8°C

Sample	The first day		24 Hrs Storage		48 Hrs Storage	
	Mean	SD	Mean	SD	Mean	SD
C1	0.000	0.000	0.10	0.06	0.1367	0.08
C2	0.000	0.000	0.09	0.08	0.54	0.07
C3	0.000	0.000	0.11	0.08	0.7	0.09
Ctrl	0.000	0.000	0.09	0.07	0.16	0.1

Key: C1= 250 µg/l chloroquine C2= 500 µg/l chloroquine C3= 1000 µg/l chloroquine Ctro= control

Table 7 shows the lysis percentage had increased when the dose of the drug was high as well as when the time of storage was prolonged.
No significant statistical difference was observed between the result of the control and blood samples processed with chloroquine.
Lysis occurred in blood samples containing each of the antimalarial drugs used in the study is inconsiderable.

Table 8

Serum sodium and potassium levels in the stored blood samples

Sample	The first day		24 Hrs Storage		48 Hrs Storage	
	Na	K	Na	K	Na	K
C1	140.70	2.833	160.10	4.4	157.8	5.267
C2	140.70	2.833	163.20	4.067	158. 5	5.633
C3	140.70	2.833	166.70	4.2	162.9	5.233
Ctrl	140.70	2.833	157.90	3.7	154.30	4.4

Key: C1= 250 µg/l chloroquine C2= 500 µg/l chloroquine C3= 1000 µg/l chloroquine
Ctro= control . Na: Sodium . K: Potassium

Table 8 shows no evident increase in serum sodium level when blood samples contain the higher dose but an increase was seen with the increase in the time of storage.
No statistical difference was detected between the results of the control and blood samples processed with 250µg/l chloroquine after 24 and 48 hours of storage nor between samples containing 500µg/l and 1000µg/l chloroquine and the control after 48 hours of storage. Serum potassium levels were increased when the dose of chloroquine was high as well as when the time of storage was prolonged.
After 48 hours no association was found between the results of the control and all blood samples. However, the upper levels of both serum potassium and sodium are within the normal range of the stored blood.

Discussion

Although polymerase chain reaction (PCR) has an increased sensitivity over blood film examination, up to14000 parasites might be present in a full unit of blood (450 ml) and still pass undetected[8]. This justifies the failure of the screening system, employed by U.S.A blood banks to prevent the occurrence of such cases[11].

Treatment of all transfused patients or prospective donors is impractical and could precipitate adverse effects. Therefore, previous studies tried to find a feasible and safe method for eradication of transfusion-induced malaria by *in vitro* processing of donors' blood with different anti-malarial drugs to provide options, since sensitivity (effectiveness of the drug to kill the parasite) is fluctuated and a certain drug may be harmful to some patients. Ali and Kadaru have tested Fansidar[12] and Quinine[13] for *in vitro* eradication of transfusion-induced malaria. The present study used chloroquine because it is a safe and cheap anti-malarial drug.

In the present study, the parasite numbers counted in samples loaded with chloroquine concentrations were significantly different from control samples. However, the determined optimal dose required for, the eradication of transfusion malaria, killing 99% of the parasites was 626.1µg/l. This dose is more than that reported by Bruce Chwatt in 1986[14] (250µg/l). This increase may be explained by resistance developed by the parasite towards the drug during the previous years and to the difference in the strains.

The mean of the prothrombin time was increased in samples loaded with the higher concentration of chloroquine, which indicates the effects of this dose on plasma coagulation factors of the extrinsic system. However, it appears that the lethal dose does not significantly affect the plasma coagulation factors of the intrinsic system since the percentage of the increase is acceptable.

The mean of the activated partial thromboplastin time correlated highly with the concentration of the applied drug increasing as the dose increased. Also, the storage duration affects the values of the APTT in samples with drugs as well as control samples (without drug). This can be explained by the presence of the labile coagulation factor VIII, which has a half-life of 24 hours. This increase of APTT is acceptable even when the blood was stored for 48 hours.

The means of the osmotic fragility values were correlated significantly with the storage time; which was increased when blood samples were stored for 48 hours compared to 24 hours. The increase of the osmotic fragility values above the control is of no significance.

Although some authors have described a relative increase in the haematocrit values of stored blood[15], these values were decreased in the present study proportional to the storage period. Nevertheless, these values were not affected by adding chloroquine; the red blood cells which had haemolysed during storage may explain the decrease of packed cell volume (PCV) in the present study.

The result of the total white blood cell count demonstrated no statistical difference, which indicates that the drugs used have no harmful effect on the viability of leucocytes.

Also, the present study revealed a significant decrease in the number of platelets, which correlated with the increase in drug concentration. However, the effect of the lethal doses on platelets appears to be acceptable. Throughout the storage, the platelet counts are within the reference values of the stored blood. These findings are in agreement with those reported by Brien J.O.R.[16] who stated that the dose of 0.1mmol/l chloroquine is too low to affect systemic platelets.

The lysis percentage in the present study was increased relative to the concentrations of the applied drug as well as the storage period. Although the highest percentage of lysis was observed in samples processed with 500 and 1000µg/l, it is low (0.7%) compared to that reported by Mollison (1%)[15].

Therefore, regardless of the mechanism by which haemolysis occurs, the survival of

processed blood stored at 4°C is within the normal range.

Storage of donors' blood in a blood bank refrigerator resulted in the increase of both sodium and potassium levels in all blood samples including the control, which is likely to be due to the halting of the active transport of these electrolytes across the red cell membrane at 2-8ºC. However, this is reversible after blood is transfused[15].

The lethal doses of the applied drug used in this study appear to be safe for all constituents of the stored blood. Chloroquine was effective and the lethal dose (626.1µg/l) can kill 99% of the parasite within 24 hours. Its application to stored blood did not produce harmful effects on the various components of the blood. Moreover, the doses did not react with the constituents of the blood bag solution (anticoagulant and preservatives) when added before blood collection. Also this procedure is inexpensive; one ampoule of chloroquine is adequate for 800 blood bags. Therefore, *in vitro* processing of donors' blood with chloroquine appears to be economical and effective.

Conclusion:

In vitro processing of donors' blood with chloroquine is a safe procedure for eradication of transfusion-induced malaria.

Chloroquine demonstrated suitable sensitivity against malaria parasites and insignificant effect on the components of the stored blood compared to the infected-control samples without chloroquine.

Future studies are recommended to select the most effective and safe antimalarial drug according to the pattern of resistance and side effects.

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