In vitro processing of donors' blood with quinine for elimination of malaria parasites

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ABSTRACT

Objective: To eliminate malaria parasites in donors' blood in vitro for eradication of transfusion-induced malaria.

Methods: We conducted the study at Ahmed Gasim Hospital in Khartoum, Sudan, between January 2005 and January 2006. Out of 4484 blood samples screened for malaria parasite microscopically, only 30 (200 ml each) satisfied the inclusion criteria of this study. The samples were subdivided equally into 4 portions. Three concentrations of quinine were separately added to 3 specimens while the fourth left without quinine (control). Blood specimens were tested on the day of collection by hematological and biochemical techniques simultaneously, and after 24 and 48 hours at 4-6ºC by the same techniques.

Results: The number of malaria parasites killed were found to be proportional to the concentrations of quinine and to the storage period, while donors' blood samples without quinine revealed a stable number of the viable parasites during the storage. Quinine was highly effective within 24 hours storage. The detected lethal dose of the applied drug to malaria parasites was generally safe to all constituents of the stored blood.

Conclusion: Our study shows that quinine could be used for the eradication of transfusion-induced malaria by in vitro processing of donors blood. The optimal doses could be added to bags' blood post phlebotomy

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Accidental inoculation of stored blood containing human plasmodium species will induce malaria. Transfusion-induced malaria, is related to clinical therapy involving the transfer of either whole blood or its components. Transfusion-induced falciparum malaria has increased in recent years, probably as it has become increasingly resistant to drugs.[1] Asymptomatic infected donors represent the source of transfusion malaria; the absence of symptoms even for a long period does not confirm the lack of infectivity.[2] Unfortunately, large numbers of parasites can be transmitted through this route of infection; whereas, most patients in need of blood transfusion, are already weakened by severe disease. Malaria thus, behaves very aggressively in such patients with higher risk of complications and fatalities.[3]

Infectivity of blood donors with malaria parasites in Sudan was 6.5% using direct microscopy and 21% using the polymerase chain reaction. This percentage is considerable, and many Sudanese patients are at a higher risk of severe malaria.[4] Many authors stressed the practical difficulties of the elimination of parasites from donors' blood in vivo before donation.[5] However, detection of malaria infection in blood donors may prove very difficult. Even by using an advanced technique, several thousand parasites present in one pint of blood may still pass undetected.[6] Moreover, it is neither ethical nor desirable to transfuse an infected

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blood to weak and ill patients, as this undoubtedly aggravates their condition. The induced disease may be difficult to control even by offering adequate doses of antimalarial drugs.[5] These facts denote the need for easy, cheap, safe, and applicable in vitro processing of donors' blood with low concentrations of quinine. These concentrations given to a patient from a single bag of transfused blood should be low compared to the complete dose that might be offered to both donors and transfused patients. Thus, this study is intended to determine the lethal dose of quinine to malaria parasite, to be added to donors' fresh blood, and to detect the hematological changes that may occur within blood stored at 4–6ºC.

Methods. The study took place in Ahmed Gasim Hospital, Khartoum State, the national capital of Sudan between January 2003 to January 2005. Three concentrations of quinine dihydrate were prepared by using 2 ml-quinine dihydrate injection, which contains 600 mg of the salt. Using the injection solution, 0.25 ml (75 mg), 0.5 ml (150 mg) and 0.75 ml (225 mg) were diluted with normal saline to obtain 15 ml of each dilution with a concentration of 5, 10 and 15 g/l respectively. Next fifty microliters of each dilution were dispensed into separate bag containing 50 ml blood; the final concentrations of each were 5, 10 and 15 mg/l.

Samples were collected between October 2002 and January 2004. All individuals who donated blood in all Khartoum state hospitals, during the study period, were included in the studied population if they satisfied the following criteria: 1. Presence of malaria parasite in donors' blood films; 2. Density of infection ranging between 1000 and 80000 parasites/ μl (only asexual stages were considered); 3. The parasites growing when cultured in the first day; 4. Donors did not receive quinine within the last 7 days, chloroquine within the last 28 days and sulfadoxine/ pyrimethamine (s/p) within the last 14 days. One pint (200 ml) (CPDA-1) of donors' malaria parasite infected blood was collected and subdivided equally into 4 prepared small bags (50 ml each) using a blood bank mixture (Biomixer-323 by). Three of these were preloaded with the 3 concentrations of quinine in addition to one (control) without drug. All blood specimens were tested for parasite culture, platelets count, total leucocyte count, packed cell volume, lysis percentage, osmotic fragility, prothrombin time, activated partial thromboplastin time, sodium and potassium serum levels, simultaneously, on the day of collection. Thereafter, it was stored in the blood bank refrigerator (4-6ºC), and tested after 24 and 48 hours by the same laboratory procedures.

Microscopic identification technique of malaria was performed as described by Cheesbrough.[7] The absolute number of parasites (number/μL) was estimated in the thin blood films by counting the parasites against 200 white blood cells, multiplied by the total leucocyte count and divided by 200. In vitro cultivation of erythrocytic stages of *Plasmodium falciparum* was intended for the assessment of the response to Fansidar and to confirm the viability/ death of the parasites. It was conducted using RPMI-1640 medium (GIBCOTM), Invitrogen Corporation. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of drug that could inhibit 95% of schizont development. It was determined by computerized probit/log dose response analysis (Statistical Package for Social Sciences [SPSS]). The indication of resistance was determined by formation of schizonts at 256 pmol or more quinine per well.

For counting the platelets and white blood cells, blood samples were diluted 1 in 20 using the (1%) ammonium oxalate solution and (2%) glacial acetic acid solutions consecutively as described by Cheesbrough.[7] Partial thromboplastin time is a screening test for the intrinsic clotting system, example, factor XII, XI, IX, VIII, X, V, prothrombin and fibrinogen. Prothrombin time is a screening test for the extrinsic clotting system, example, factor VII, X, V, prothrombin and fibrinogen. These tests were performed as described by Dacie and Lewis[8] using the reagent of DiaMed Company, Switzerland.

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. This was carried out as described by Dacie and Lewis.[8]

Packed cell volume is the proportion of whole blood occupied by red cells, which were measured by the micro-hematocrit method described by Dacie and Lewis.[8] The percentage of lysis is a real indicator for survival of red blood cells during the storage period. Five ml of each blood sample, just after collection, were centrifuged for 5 minutes at 12000 g. The supernatant was stored sterile at 4°C to be used as blank. Five hundred microliters of the precipitated red cells were diluted in 5 ml of pure distilled water and stored at 4°C to be used as standard. Both solutions were used in the determination of lysis percent for the same sample throughout the storage period. One ml of each blood sample after 24 and 48 hours was also centrifuged for 5 minutes at 12000 g and the supernatant of each was tested for hemolysis at 540 nm wavelength. The 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, England. Data were analyzed using the SPSS program. Non-Parametric tests were mainly used for the abnormal distribution of the data. Data were analyzed by the 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 were 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 were 4484, which were subjected to screening for malaria parasite microscopically using standard Giemsa' staining technique. A total number of 278 (6.2 %) blood bags' specimens were found to carry malaria parasite. The parasite densities were less than a thousand in 215 (77%), while more than a thousand parasite-densities were observed in 63 (22.7%) specimens. Of these, 30 (10.8%) samples were found to be resistant to chloroquine in addition to another 3 (9.09%) samples showing no growth when cultured in the first day. Thus, the actual number of blood samples for this study was reduced according to the parameters previously-mentioned, to only 30. However, all of the accepted blood bags belonged to asymptomatic male donors between 25 and 35 years old.

Parasites' count decreased with the increasing concentration of quinine and elongation of storage period. Thus, the parasites disappear after 24 hours in blood samples with the higher concentration (15 mg/L), and after 48 hours in samples with the medium concentration (10 mg/L). The donors' blood control samples (without addition of antimalarials) reveal stable number of the parasites even after 48 hours storage. Hence, the lethal doses were observed to be high (7.98 mg/L) when donors' blood was stored for 24 hours. The lethal doses were lower (4.88 mg/L) when the blood was stored for 48 hours.

In **Table 1**, prothrombin time and partial thromboplastin time were increased with the increase of the quinine dose and duration of storage. Prothrombin time reached the peak in samples with a concentration of 10 and 15 mg/L, while minimum increase was observed when the minimum concentration was added. On the other hand, partial thromboplastin time reached the peak in samples with a concentration of 10 and 15 mg/L. Minimum increase was observed when the minimum concentration of all drugs was added. Although there is a significant statistical difference between the results of both prothrombin time and partial thromboplastin time among different blood samples (when stored for the same time), still the upper levels are within the normal range.

In **Table 2**, it is observed that osmotic fragility values had increased in samples with higher doses of quinine as well as prolongation of 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 were stored for 48 hours, correlation between processed blood samples and the control reveal statistical similarity in samples with 5 mg/L and differences in those with a concentration of 10 and 15 mg/L; nevertheless, the upper levels remain within the normal. Also, the table demonstrates the decrease of packed cell volume when the time of storage was prolonged regardless of the increase of the dose. No significant statistical difference was observed between the results of the control, and blood samples during the storage period.

Table 3 illustrates that the total leucocyte count, and platelets numbers decreased with the increase in storage time but they were not correlated with the dose concentration. No significant statistical difference was observed between the results of the control and

Table 1 - Prothrombin and partial thromboplastin times (in seconds) during the storage period.

Sample	The first day		24 hrs incubation		48 hrs incubation	
	PT	APTT	PT	APTT	PT	APTT
Q1	15.67	36.2	16.4	38.97	18.8	42.8
Q2	15.67	36.2	17	42.17	19.8	45.53
Q3	15.67	36.2	17.53	44.03	20.5	49.07
Control	15.67	36.2	15.93	38.4	17.97	40.867

Q1 - 5 mg/L quinine, Q2 - 10 mg/L quinine, Q3 - 15 mg/L quinine, PT - prothrombin time, APTT - activated partial thromboplastin time

Table 2 - Means of osmotic fragility and hematocrit values of blood samples stored at 2-8°C.

Sample	The first day		24 hrs incubation		48 hrs incubation	
	OFT	PCV	OFT	PCV	OFT	PCV
Q1	0.451	42.7	0.465	36.1	0.505	36.2
Q2	0.451	42.7	0.475	38.6	0.495	38.3
Q3	0.451	42.7	0.475	36.9	0.51	37.9
control	0.451	42.7	0.46	40.5	0.46	39.5

Q1 - 5 mg/L quinine, Q2 - 10 mg/L quinine, Q3 - 15 mg/L quinine, OFT - osmotic fragility, PCV - hematocrit

all of the processed blood samples throughout the storage period.

Table 4 demonstrates the means and standards deviations of the lysis amount in the control and processed blood samples on the day of collection, after 24 hours and 48 hours storage at 2-8°C. Lysis percentage had increased after 24 hours when the dose of the drug was high as well as when the time of storage was prolonged. Forty-eight hours storage result in the increase of lysis percentage as the time of storage was prolonged. A significant statistical difference was observed between the result of the control and blood samples processed with the higher dose (15 mg/L) only; whereas, no statistical difference was detected between the control and the other processed blood samples. Lysis in blood samples loaded with all of the applied concentrations is inconsiderable.

Table 5 illustrates the means and standard deviations of the serum sodium and potassium levels in the control and processed blood samples on the day of collection, after 24 hours and 48 hours storage at 2-8°C. Serum sodium level increase was not evident in blood samples loaded with the higher dose, while the increase of the time of storage has led to an elevation in case of the first (Q1) and third (Q3) doses. Serum potassium levels were increased with the increase of the storage time regardless the dose.

A significant statistical difference was observed between the results of the control and blood samples processed with all doses. However, the upper levels of both remain within the normal range of the stored blood.

Table 3 - Total white blood cells and platelets counts during the storage period.

Sample	The first day		24 hrs incubation		48 hrs incubation	
	TWBC	PLTS	TWBC	PLTS	TWBC	PLTS
Q1	3250	225000	2490	17500	2510	157000
Q2	3250	225000	3110	163700	2610	148000
Q3	3250	225000	3200	163000	3970	147000
Control	3250	225000	2940	208000	3230	178000

Q1 - 5 mg/L quinine, Q2 - 10 mg/L quinine, Q3 - 15 mg/L quinine, TWBC - total white blood cells count, PLTS - total platelets count
*Values are cells per microliter

Table 4 - The lysis percentage of the stored red blood cells during the 48 hours storage.

Sample	The first day	24 hrs incubation	48 hrs incubation
	Mean ± Std	Mean ± Std	Mean ± Std
Q1	0	0.153 ± 0.08	0.2 ± 0.07
Q2	0	0.154 ± 0.07	0.17 ± 0.08
Q3	0	0.169 ± 0.09	0.2 ± 0.08
Control	0	0.09 ± 0.07	0.16 ± 0.10

Q1 - 5 mg/L quinine, Q2 - 10 mg/L quinine, Q3 - 15 mg/L quinine, Std - standard deviation

Table 5 - The levels of serum sodium and potassium in the stored blood samples.

Sample	The first day		24 hrs incubation		48 hrs incubation	
	Na	K	Na	K	Na	K
Q1	140.7	2.833	170.3	4.167	187.7	5.3
Q2	140.7	2.833	169.2	4.633	165.1	5.033
Q3	140.7	2.833	166.9	4.267	167.26	4.767
Control	140.7	2.833	157.9	3.7	154.3	4.4

Q1 - 5 mg/L quinine, Q2 - 10 mg/L quinine, Q3 - 15 mg/L quinine, N - serum sodium level, K - serum potassium level
*Values are in mmol/L

Discussion. Transfusion-induced malaria is related to accidental inoculation of stored blood containing human plasmodium species. Screening blood donors for malaria parasites will minimize, though never eliminate the risk.[9] The task of routine detection of malaria parasites in blood donors is difficult for 3 reasons: 1. their numbers in the peripheral blood are scanty and very often below the threshold of microscopic examination; 2. frequent absence of any symptoms including fever; and 3. unavailability of advance rapid techniques.[5] Although PCR has an increased sensitivity over blood film examination, with a detection limit of 0.004-0.04 parasites per microliter, 1400-14000 parasites might be present in a full unit of blood (450 ml) and still pass undetected. This explains the failure of the screening system, employed by the United States blood banks to prevent the occurrence of such cases.[6] It is not desirable to treat all transfused patients who are already suffering from variable diseases, and taking a lot of drugs other than antimalarials. The drugs taken by the patient may precipitate serious side effects with a critical, if not fatal, prognosis. Therefore, it is essential to process donors' blood in vitro to ensure the effectiveness of the procedure in killing the parasite before transfusing the blood.

In the present study, a significant statistical difference was detected between the numbers of

positive samples when loaded with the 3 doses, thus, reflecting the effectiveness of the drug proportional to their concentrations. The lethal doses determined in the present study, were found to be 7.9 mg/L; it kills 99% of the parasites in maximum 24 hours storage. These doses are more than that reported by Chwatt[10] in 1986[10] (5 mg/L). This increase is likely to be due to the behavior of the parasite towards the drug during the previous 19 years, which resulted in adaptation of the parasite to become in relative resistance. Also, the difference in the strains may explain this increase. However, Ackerman et al[11] used quinine solution at 1:5000 (0.2 mg/L) for the same purpose but they did not detect any lethal effect. This failure can be explained by the small concentration of the applied drug, which is rather low to kill the parasite.

The time of storage affects both of the prothrombin time (PT) and activated partial thromboplastin time (APTT) values in samples loaded with the drug as well as control samples (without drug). This may be justified by the presence of the labile coagulation factor VIII, which has a half-life of 8-12 hours. However, the increase of PT and APTT in all samples with the recognized lethal doses is acceptable even when the blood was stored for 48 hours. The means of the osmotic fragility values are significantly correlated with the storage time; they had increased when blood samples were stored for 48 hours rather than those stored for 24 hours. However, it appears that the lethal dose of quinine has no effects on the flexibility of the cell membrane; since no significant statistical difference was observed.

Some authors have described a relative increase in the hematocrit (PCV) values of the stored blood by the increase of storage time.[12] On the contrary, PCV values in the present study were decreased proportionally to the prolongation of the storage period. The red blood cells hemolyzed during the storage may explain the decrease of PCV. In order to evaluate the action of the drug on the viability of white blood cells, the different blood samples included in the study were subjected to a total white blood cell count. The result revealed no statistical difference, indicating that the used drugs have no harmful effect on the number of leucocytes. However, the effect of the lethal dose on platelets appears to be acceptable; its number is within the reference values.

The lysis percentage among the stored blood reflects the unfavorable effect of storage or applied drugs on the cell membrane, red cell enzymes or hemoglobin nature. The lysis percentage in the present study has increased proportionally to the concentration of the applied doses as well as the storage period. The highest percentage of lysis observed in samples is far low (0.7%) compared to that reported (1%) by Mollison.[12] Therefore, regardless of the mechanism by which hemolysis occurs; the survival parameters of processed blood stored at 4°C are within the normal range.

Storage of donors' blood in bank refrigerator results in the increase of both sodium and potassium levels, which is likely to be due to the halt of the active transport of these electrolytes across the red cell membrane. In the present context, the samples have showed poor correlation between sodium levels and drug concentration despite its good correlation with the storage period. These facts do not match the result of potassium levels. It did not correlate with the concentration of the applied drug while it is closely correlated with the duration of the storage. However, the increase in the percentage of the potassium level is acceptable when blood samples were processed with the lethal dose.

It appears that quinine is most effective for in vitro processing of donors' blood. Its application to stored blood did not produce harmful effects on the different constituents of the blood. Moreover, addition of more lactose to the blood subjected for quinine application is recommended since quinine reduces the concentration of blood glucose.[12] Further, this procedure is inexpensive; one ampoule of quinine is satisfactory for the treatment of 150 blood bags. Therefore, compared to the entire provided solutions, in vitro processing of donors' blood with antimalarial drugs appears to be a merit.

In conclusion, In vitro processing of donors' blood with quinine could be the safest procedure applied for eradication of transfusion-induced malaria. Quinine revealed high sensitivity to malaria parasites and insignificant effect on the components of the stored blood compared to the infected-control samples to which antimalarial drugs were not added. Future studies are recommended to select the highly effective and safe antimalarial drug according to the pattern of resistance and side effects.

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