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A cost-effective scheme developed for studying human malaria caused by Plasmodium falciparum

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Abstract
Research in human malaria disease has consistently been hindered in developing countries where this disease is endemic, due to the prohibitive cost of constructing and maintaining currently available experimental mouse models. Our goal, therefore, was to develop a cost-effective mouse model that may be used as research tool for studying human malaria disease. *Plasmodium falciparum*-infected human blood samples were cultured invitro for 92 hours, and invivo malaria infection was induced by intraperitoneally injecting 0.5ml of the *Plasmodium falciparum* cultures into experimental mice, which were modified by the application of immunosuppressive and humanization protocols in which aspirin (4mg/kg), doxycycline (4mg/kg), and 0.5ml human blood that retained all of its cellular components (erythrocytes, leukocytes, and platelets) were repeatedly injected via the intraperitoneal route. Data obtained showed that the invitro-cultured *Plasmodium falciparum* significantly retained its infectivity and immunogenicity, since all the 20 mice inoculated exhibited peripheral blood parasitaemia. Quinine chemotherapy using standard antimalarial drug (73mg quinine/kg), however, induced significant suppression of the peripheral blood parasitaemia in the infected mice. Our results suggest that there is a substantial possibility of inducing and eradicating human malaria disease in our mouse model (humanized non-genetically manipulated mouse model) when used as a substitute for the conventional mouse models (humanized genetically manipulated mouse models).
Keywords: Synchronized, Invitro, Invivo, Inocula, Immunosuppressed, Parasitaemia, Intraperitoneal, Infectivity.

1. Introduction

Malaria is an ancient disease that continues to cause enormous human morbidity and mortality. It is caused by the erythrocyte stage of protozoan parasites of the genus - *Plasmodium*. Besides the challenges of multi-drug resistance posed by *P. falciparum*, a significant burden inhibiting the development of malaria research is the dearth of experimental non-human species for invivo studies of human-competent *P. falciparum* (Jimenez-Diaz *et al*., 2009). Currently, laboratory mice are the only non-human models available for studying *P. falciparum* biology inside human erythrocytes.

In studies conducted so far (Moreno *et al*., 2001; Arnold *et al*., 2011), mouse models used for human malaria research were constructed by the application of immunosuppression and humanization protocols. However, the cost implication of acquiring and maintaining the immunosuppressed mouse models currently available has retarded research activities in human malaria disease, especially in developing countries. The high cost encountered in constructing these immunosuppressed mouse models arises from: the highly specialized task involved in manipulating the genetics of the mouse strains; the non-easily accessible prescriptive pharmacological agents, for example, neutrophil marker monoclonal antibody (NIMP-R14), dexamethasone and cyclophosphamide, employed to complementarily inhibit innate defenses of the immunsouppressed mouse strains; as well as the specialized maintenance protocols required to sustain these mouse strains (Jimenez-Diaz *et al*., 2009; da Costa de Avila *et al*., 2012).

Previous studies have also shown that relatively inexpensive easily accessible across-the-counter pharmacological agents, such as aspirin and doxycycline, exhibit immunosuppressive activities (Zhu *et al*., 2007; Leite *et al*., 2011). However, our study is, arguably, the first attempt to synergize the immunosuppressive effects of these two inexpensive easily accessible across-the-counter pharmacological agents (aspirin and doxycycline) to solely inhibit the innate defenses of non-genetically manipulated mouse strains; and to subsequently humanize the resultant immunosuppressed mouse strains by the engraftment of human blood that retained all of its cellular components (erythrocytes, leukocytes, and platelets). Our study, therefore, aims to develop a relatively inexpensive humanized non-genetically manipulated mouse model as a tool for studying human malaria disease.

2. Materials and Methods

2.1 Invitro culture of Plasmodium falciparum

2.1.1 Collection of human blood samples

The human blood samples that showed malaria infection upon preliminary screening were collected from University of Benin Teaching Hospital (UBTH), Nigeria. Patients' samples were collected following written informed consent, and ethical approval was obtained from the Igbinedion University Ethics Committee.

2.1.2 Invitro culture set-up

Invitro cultures were set up according to previously described standard procedure (Trager and Jensen, 1976), with slight modifications. Parasites were grown in group A^+ heparinized blood cells (Igbinedion University Teaching Hospital (IUTH), Nigeria) in RPMI 1640 prototype medium (specially formulated by the authors of the present study) supplemented with 25mM $NaHCO_3$ (BDH, England), 10mM glucose (BDH, England), 5.3ppm gentamycin (Greenfield Pharmaceutical Ltd, China), and 10% human serum (IUTH, Nigeria). Parasites were maintained at 5% haematocrit in the complete medium and incubated at room temperature (28 ± 2^oC) in candle jars for 46 hours.

2.1.3 Synchronization of cultures after 46 hours incubation

Synchronization was performed according to previously described standard method (Lambros and Vandenberg, 1979). The infected cells and 5% sorbitol (Qualikems, India) were mixed using a dilution factor of 10, and kept at room temperature for 5 minutes, after which treated cells were washed thrice with complete medium by centrifugation (Centrifuge; PEC Medical, USA) at 400 x g for 4 minutes at room temperature.

2.1.4 Invitro subculture set-up

Invitro subcultures, which utilized the 46 hours synchronized cultures as inocula, were set up following similar procedure in 2.1.2. The subcultures were incubated for another 46 hours.

2.1.5 Synchronization of subcultures after 46 hours incubation

Subcultures were synchronized according to the method stated in 2.1.3.

2.1.6 Dilution of synchronized subcultures

Each synchronized subculture was diluted by mixing the culture with the complete medium and uninfected group A^+ blood cells, after which, it was used as an inoculum to induce infection in the experimental mice.

2.1.7 Evaluation of invitro cultures

Blood films of cultures were examined for parasites according to the method of World Health Organization (WHO, 2009). Parasite count per microliter (µl) of blood was calculated according to this formula:

$$Number\ of\ parasites/\mu l = \frac{Number\ of\ parasites}{Number\ of\ leukocytes\ counted} \times white\ blood\ cell\ count/\mu l$$

Parasitaemia was also expressed as the percentage of red blood cells infected. 1% parasitaemia represented 50,000 parasites /µl of blood (WHO, 2009).

2.2 Development of mouse model

2.2.1 Mice

Non-genetically manipulated mice (21 - 31g) of both sexes were purchased from the Animal House in the University of Benin, Benin City, Nigeria. The mice were housed in standard mosquito-netted metal cages under standard conditions of light and temperature in the Animal Housing facility at Igbinedion University, Okada (IUO). They were maintained on standard diet and water *ad libitum*. The mice were acclimatized for 7 days and were treated in accordance with guidelines for animal care approved by the Animal Ethics Committee of the Igbinedion University. The mice were certified medically fit for the experiment by Dr. J. Danjuma, a veterinary doctor.

2.2.2 Immunosuppression of mice

Immunosuppression was carried out by an initial treatment (pre-treatment) of non-genetically manipulated mice with aspirin (4mg aspirin/kg body weight; Juhel Nig. Ltd, Nigeria) and doxycycline hydrochloride (4mg anhydrous doxycycline eq. /kg body weight; Vapi Care Pharm. Pvt. Ltd., India) for 20 days prior to humanization. Upon humanization, the resultant humanized immunosuppressed mice were repeatedly treated with similar dosages of aspirin and doxycycline throughout the duration of the study, and were only terminated upon demise of the mice. Aspirin and doxycycline were intraperitoneally injected into the mice.

2.2.3 Humanization of immunosuppressed mice

The immunosuppressed mice were initially engrafted with group O^+ blood cells (0.5ml, 50% haematocrit) for 6 days, followed by a repeated engraftment with group A^+ blood cells (0.5ml, 50% haematocrit) for another 5 days. Upon inoculation of mice with *P. falciparum* cultures, mice were also daily transfused with group A^+ blood cells throughout the duration of the study, and were only terminated upon demise of the mice. The human blood cells were intraperitoneally injected.

2.3 Infection of mouse model

0.5ml diluted synchronized subcultures of *P. falciparum* served as inocula for infection of mice. Inocula were injected intraperitoneally into the mice after 11 days of engraftment with group O^+ and A^+ blood cells. Diluted subculture obtained from Sample A was inoculated into each mouse in test group 1; diluted subcultures obtained from Samples B, C, and D were also inoculated into each mouse in test groups 2, 3, and 4 respectively. Mice in the control group were not inoculated.

2.3.1 Examination of blood films

Thick and thin blood films of the cardiac and venous blood were examined for *P. falciparum* parasites according to the method of World Health Organization (WHO, 2009).

2.4 Validation of the new mouse model for antimalarial chemotherapy studies

Quinine was administered according to previously described standard procedure (Moreno *et al*., 2001). *P. falciparum*-infected mice that survived the malaria infection were divided into control and test groups respectively. Each mouse in the control group received an oral dose (4mg/kg) of placebo [dimethyl sulfoxide (DMSO)] three times daily for four days. Each mouse in the test group also received an oral dose (73mg/kg) of quinine three times daily for four days.

2.4.1 Evaluation of antimalarial chemotherapy protocol

Upon administration of quinine and DMSO, parasite densities were obtained from mice that either died or survived during the chemotherapy phase according to the method of World Health Organization (WHO, 2009). Net mean chemosuppression due to drug administration was calculated according to the formulation of Bassey *et al*. (2009), with slight modifications:

$$\text{Net mean chemosuppression (\%)} = \frac{100\,(A - B)}{A} - MC0$$

Where,

A = Percentage of parasitaemia obtained in the mice administered with DMSO

B = Percentage of parasitaemia obtained in mice treated with quinine

$\frac{100\,(A - B)}{A}$ = Mean chemosuppression

MC0 = Mean chemosuppression obtained from mice at day 0 of treatment.

2.5 Statistical analysis

All the values were expressed as mean and standard error of the mean. Chi-square test and regression analysis, where appropriate, were used to determine the level of significance, and P-value less than 0.05 ($P < 0.05$) were considered significant. The software SPSS version 16 was employed for the statistical analysis.

3. Results

3.1 Invitro culture of Plasmodium falciparum

Table 1 represents parasitaemia obtained from the inocula, 92 hours synchronized invitro cultures, as well as their growth factors (degree of parasitaemia). The morphology of *P. falciparum* obtained from the invitro culture system is shown in the photomicrographs in Figure 1.

3.2 Infection of mouse model

Table 2 represents peripheral blood parasitaemia obtained from 25 humanized immunosuppressed mice which survived the first phase of this study (development of mouse model) and were, thus, selected for the infection phase. Figure 2 compared the parasitaemia obtained from each of the groups infected with each of the different human blood samples. Figure 3 represents photomicrographs of the peripheral blood of humanized immunosuppressed mice that died of malaria infection caused by *P. falciparum*, while Figure 4 shows photomicrographs of the peripheral blood of humanized immunosuppressed mice that survived malaria infection caused by *P. falciparum*.

3.3 Antimalarial chemotherapy

Table 3 represents parasitaemia and mean chemosuppression obtained from 12 *P. falciparum*-infected mice that survived the second phase (infection phase) of experiment, and were, thus, selected and used for the antimalarial chemotherapy validation phase. The effect of quinine/DMSO therapy on parasite suppression in the *P. falciparum*-infected mice is analysed in Figure 5.

4. Discussion

Our study describes a new approach of modifying mice to carry out studies on human diseases (with emphasis on malaria disease caused by human-competent *Plasmodium falciparum*).

The invitro culture system employed in our study (Table 1) had significant similarities with the types used by other authors (Trager and Jensen, 1976; Moreno *et al*., 2001; Arnold *et al*., 2011). By using a non-genetically

manipulated mouse model, our study introduces a markedly different approach from previous studies (Moreno *et al*., 2001; Jimenez-Diaz *et al*., 2009; Arnold *et al*., 2011) which mainly utilized genetically manipulated mouse models as research tools for human malaria research. To optimally control inflammation in our mouse model, we employed pharmacological compounds (aspirin and doxycycline) that were also markedly different from the conventional compounds (neutrophil marker monoclonal antibody (NIMP-R14), dexamethasone, and cyclophosphamide) used in previous studies.

The high parasite densities (Table 2) associated with the severe malaria infection may have overwhelmed the phagocytotic and necrotic effects of neutrophils (Arnold *et al*., 2011), thus, resulting in the sequestration of the *P. falciparum* parasites, and the ultimate death of some infected mice. In spite of the enormous innate defenses exerted in the peritonea of the mice inoculated with *P. falciparum* cultures, results obtained from our study showed that all the 20 mice inoculated with *P. falciparum* cultures at day 31 of experiment produced peripheral blood parasitaemia (Figure 2), indicating that some of the parasites survived by successfully circumventing the innate defenses mounted by peritoneal phagocytes. Parasite survival may be due to a significant attenuation of inflammatory responses, as a result of repeated intraperitoneal injection of the immunosuppressive drugs (Arnold *et al*., 2010). The receptivity by our immunosuppressed mice of human erythrocyte component of the blood that were repeatedly injected into their peritonea, which provided a suitable habitat and nutrient for the parasites, may have also accounted for the parasites' survival. Unlike the invitro culture system [Figure 1 (B and C)], where there was absence of spontaneous synchrony of the cultured *P. falciparum*, the invitro-cultured *P. falciparum* parasites, that entered the peripheral blood of mice employed in our study, exhibited remarkable spontaneous synchrony [Figure 3 (B and C); Figure 4 (A and B)]. Synchrony was, however, induced in the invitro culture system by treatment with 5% sorbitol. As indicated above in our study, all the results obtained from the infection phase significantly correlated with those recorded in humans (Arnold *et al*., 2011), thus, alluding to the potential immense value of this new mouse model for studying other human diseases.

The new mouse model was validated for antimalarial chemotherapy research. Upon quinine therapy, our new mouse model showed significant apoptosis ($P < 0.05$) of *P. falciparum* parasites in the blood of peripheral and deep-seated capillaries when compared to DMSO therapy (Table 3; Figure 5), thus, significantly conforming to those obtained from previous studies (Moreno *et al*., 2001; Arnold *et al*., 2011).

5. Conclusions

Our study suggests that there is a substantial possibility of inducing and eradicating human diseases by using our mouse model, whose components of innate and adaptive immunity have been substantially substituted with those in humans through the repeated intraperitoneal injection of human blood that retained its leukocytes. Our results, therefore, corroborate the assertion of Arnold *et al*. (2010), who stated that "a deficiency in adaptive immunity is far from being enough to ensure the success of xenografts".

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Table 1. Parasitaemia obtained from the invitro culture system

Human blood Samples	Parasite counts						Growth factors
	Inocula		92 hours synchronized cultures		Diluted 92 hours synchronized cultures		
	($\times 10^3$cells/µl)	(%)	($\times 10^3$cells/µl)	(%)	($\times 10^3$cells/µl)	(%)	
A	24	0.48	2,040	40.8	400	8	85
B	36	0.72	2,003	40.06	400	8	56
C	33.2	0.66	1,941	38.82	400	8	59
D	20.64	0.41	1,796	35.91	400	8	87

As recorded in Table 1, varying parasite densities (0.41% to 0.48%) were obtained from the four different *P. falciparum*-infected samples (A – D) used as inocula for the invitro cultures. After 92 hours of incubating synchronized cultures of the blood samples, parasite densities (35.91% to 40.8%) obtained from the invitro cultures were markedly different from values of their inocula. Sample D had the highest growth factor (87- fold increase), while the lowest growth factor (56-fold increase) was recorded in sample B. All the synchronized invitro subcultures were, however, diluted using different dilution factors (5.1, 5, 4.85, 4.51 for Samples A, B, C, and D respectively), so as to obtain invitro cultures with the same parasite densities (8%), from which equivalent volume (0.5ml) were used as inocula for invivo infection of mice.

Table 2. Parasitaemia obtained from immunosuppressed mice infected with *P. falciparum*

Days of infection	Mice	Inoculum employed	Mean parasite counts ($\times 10^3$ cells/µl)	Mean parasite counts (%)	Overall mean parasite counts ($\times 10^3$ cells/µl)	Overall mean parasite counts (%)
0	Control group	None (-)	0 ±0	0 ±0	0 ±0	0 ±0
	Test group 1	Sample A	0 ±0	0±0	0±0	0±0
	Test group 2	Sample B	0 ±0	0±0		
	Test group 3	Sample C	0 ±0	0±0		
	Test group 4	Sample D	0 ±0	0±0		
2	Control group	-	0 ±0	0±0	0±0	0±0
	Test group 1	*	13.5 ±0.69	0.27 ±0.01	18.8 ±4.2	0.38 ±0.08
	Test group 2	*	16 ±0.62	0.32±0.01		
	Test group 3	*	31.3 ±14.2	0.63 ±0.28		
	Test group 4	*	14.4 ±0.68	0.29 ±0.01		
4	Control group	-	0±0	0±0	0±0	0±0
	Test group 1	*	55.4 ±16.8	1.11 ±0.34	43.8±5.97	1±0.17
	Test group 2	*	43.1 ±3.94	1.36 ±0.57		
	Test group 3	*	49.2 ±1.39	0.99 ±0.03		
	Test group 4	*	27.6 ±2.76	0.55 ±0.05		
6	Control group	-	0±0	0±0	0±0	0±0
	Test group 1	*	64.6 ±24.3	1.29 ±0.49	48.2±6.96	0.97±0.14
	Test group 2	*	39.4 ±5.06	0.79 ±0.1		
	Test group 3	*	54.5 ±0.22	1.1 ±0.01		
	Test group 4	*	34.3 ±1.64	0.69 ±0.03		

(*) indicates that mice were only inoculated with the invitro *P. falciparum* cultures at day 0 of infection. All values represent mean ± standard error mean (SEM).

Unlike in the control group (Table 2), where the mice remained uninfected (mean parasite count at day 0 to 6 of infection = 0%), all the mice in the test groups (Table 2) were markedly infected, as indicated by the overall mean parasite counts (0%; 0.38±0.08%; 1±0.17%; 0.97±0.14%) obtained at days 0, 2, 4 and 6 of infection respectively.

Table 3. Parasitaemia obtained from infected mice treated with quinine and dimehyl sulfoxide

Days of therapy	Mice	Drugs admin-istered	Mean parasite counts ($\times 10^3$ cells/µl)	Mean parasite counts (%)	Mean chemo-suppression (%)	Net MC (%)
0	Control group	DMSO	77.05±3.18	1.54±0.06	1.19	0
	Test group	Quinine	76.14±4.45	1.52±0.09		
2	Control group	DMSO	80.79±2.6	1.62±0.05	60.67	59.49
	Test group	Quinine	80.95±1.28	0.64±0.03		
4	Control group	DMSO	83.58±15.94	1.67±0.32	83.82	82.65
	Test group	Quinine	13.52±0.57	0.27±0.01		

Net MC represents net mean chemosuppression; DMSO represents dimethyl sulfoxide.

In the group of mice treated with quinine (Table 3), parasite counts were estimated at 1.52±0.09% and 0.27±0.012% at days 0 and 4 of therapy respectively; while in the group treated with DMSO (Table 3), parasite counts were estimated at 1.542±0.063% and 1.672±0.32% at days 0 and 4 of therapy respectively.

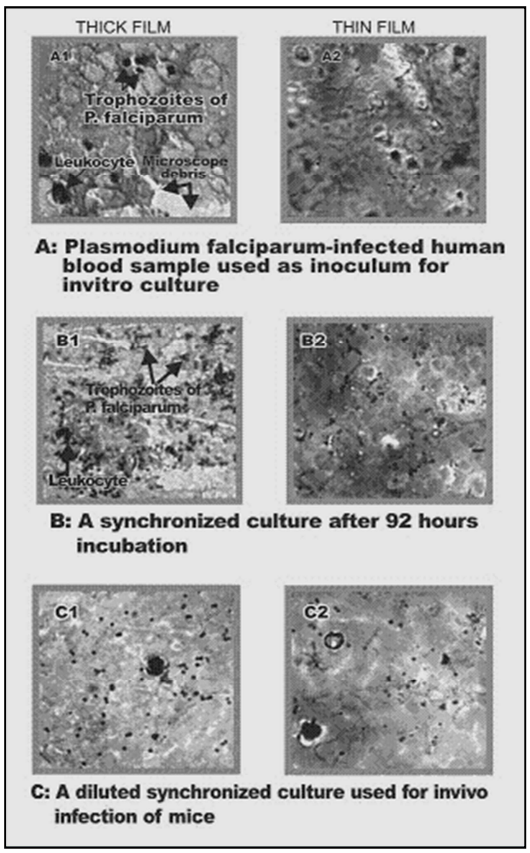

Figure 1. Photomicrographs obtained from invitro culture of *Plasmodium falciparum*

In Figure 1A, the photomicrograph (Sample A) of the *P. falciparum*-infected human blood sample showed a predominance of matured *P. falciparum* trophozoites, lymphocytes, and monocytes. The photomicrographs of other samples (Samples B, C, and D) had similar patterns with that shown in Figure 1A. Photomicrograph of the synchronized invitro culture obtained from Sample A, after 92 hours incubation, is shown in Figure 1B. It showed exponential proliferation of the parasites, with a vast majority being at the same stage of growth. The other samples (B, C, and D) also exhibited similar patterns with that shown in Figure 1B. Figure 1C represents photomicrograph of a diluted synchronized invitro culture obtained from Sample A, which was used for the infection of experimental mice in test group 1.

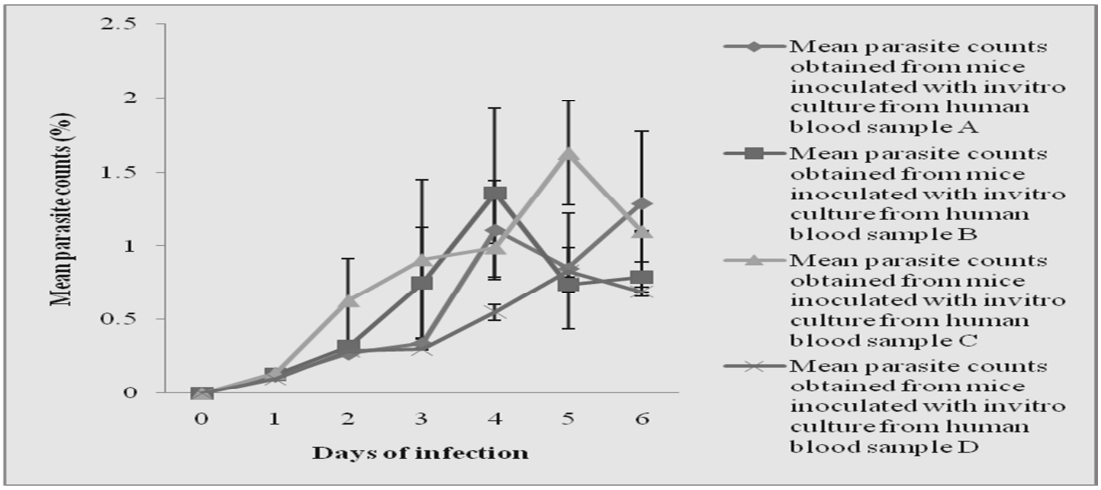

Figure 2. Invivo parasitaemia patterns exhibited by cultures obtained from the different clinical blood samples which were each respectively used as an inoculum to infect 5 humanized immunosuppressed mice in each of the experimental group

As shown in Figure 2, the mice in group 3 inoculated with synchronized cultures obtained from blood sample C had the highest parasite counts, with peak parasitaemia level attaining 1.63% at day 5 of infection; while the least parasite counts were recorded in the mice in group 4 (parasitaemia level = 0.68% at day 6 of infection) which were

inoculated with synchronized cultures obtained from blood sample D. There were large variations in the parasite densities of some mice in each of the groups infected with invitro cultures obtained from the different human samples, as indicated by the values of their standard errors. These variations arose from the very high parasite counts obtained from the cardiac blood films of the mice that died of malaria infection, as compared to the relatively low parasite counts obtained from the venous blood films of mice that survived the malaria infection phase in each experimental group.

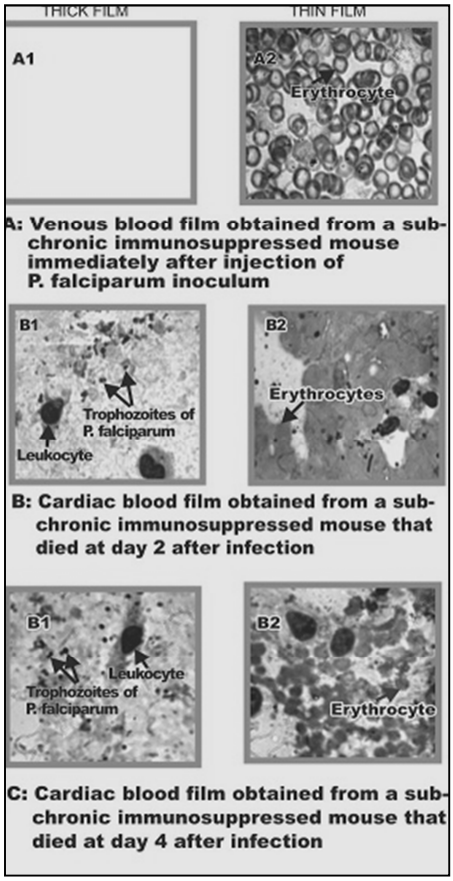

Figure 3. Photomicrographs of the peripheral blood of humanized immunosuppressed mice that died of malaria infection caused by *P. falciparum*

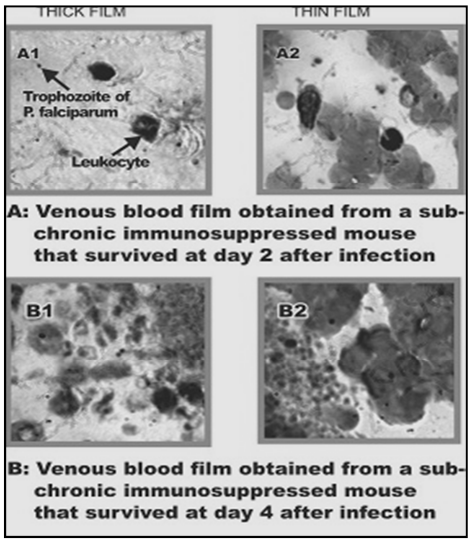

Figure 4. Photomicrographs of the peripheral blood of a humanized sub-chronic immunosuppressed mouse that survived malaria infection caused by *P. falciparum*.

When the photomicrograph obtained from mice that died of malaria infection caused by *P. falciparum* at day 2 and 4 after infection [(Figure 3 (B and C)] were compared to that obtained from a mouse that were injected with *P. falciparum* at day 0 of infection (Figure 3A), the micrographs showed that the peripheral blood of infected mice

were morphologically altered. Photomicrograph obtained from infected mice that survived the infection phase, as shown in Figure 4 (A and B), also showed a morphologically altered peripheral blood. As shown in Figure 3A, significant proportions of intact erythrocytes were seen in the peripheral blood of the humanized immunosuppressed mice immediately after they were intraperitoneally injected with malaria parasites. However, upon infection, cardiac and venous blood films obtained from infected mice that either died [Figure 3 (B and C)] or survived [Figure 4 (A and B)] revealed a proliferation of lymphocytes, monocytes, and trophozoites of *P. falciparum* that were largely at the same stage of growth; though, relatively low parasite densities were seen in the venous blood of mice that survived, when compared to those seen in the cardiac blood of mice that died of malaria infection. Remarkably, the photomicrographs shown in Figure 3 (B and C) and Figure 4 (A and B) obtained from mice significantly resembled the photomicrograph shown in Figure 1A obtained from a human blood sample infected with *P. falciparum*.

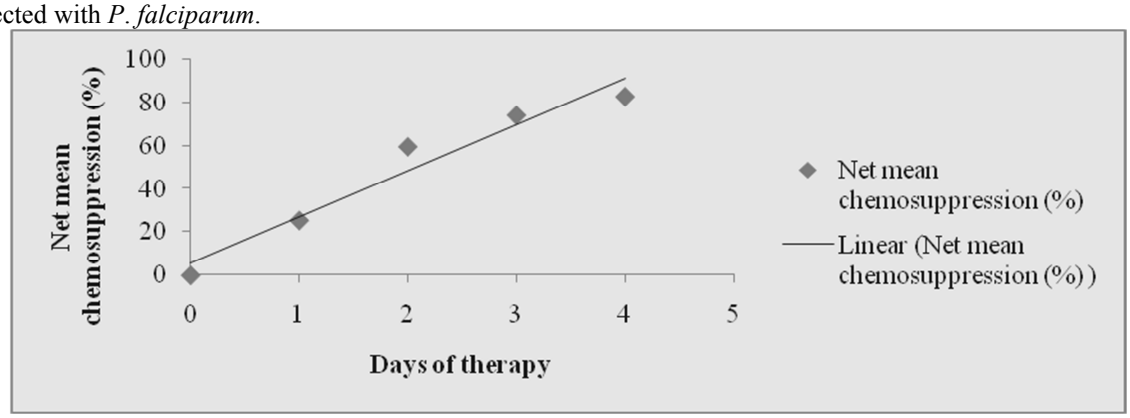

Figure 5. Effect of antimalarial therapy on *P. falciparum* suppression in the infected immunosuppressed mouse model ($r= 0.98$, $P < 0.05$)

According to the regression graph shown in Figure 5, quinine was well tolerated in the new mouse model, and exhibited a highly potent effect, by significantly clearing ($P < 0.05$) the parasitaemia of the infected mice in the test groups, as indicated by net mean chemosuppression of 0% and 82.65% at days 0 and 4 of antimalarial therapy respectively.