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Original Article

Interaction between Chitosan and Chloroquine against *Plasmodium berghei* and *P. falciparum* using In-Vivo and In-Vitro Tests

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Abstract

Background: The use of antimalarial drugs with number of compounds in combination form may potentiate each other's activity.

Methods: This study was conducted in the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran in 2018. It was based on two methods including in vivo and in vitro tests with aim of considering interaction between chitosan and chloroquine against *Plasmodium berghei* and *P. falciparum* parasites using different ratios of the agents with ED50^s and IC50^s baselines.

Results: Administering 10 and 20 mg/kg (mouse body weight) of chitosan alone to the *P. berghei* -infected mice up to 4 successive days resulted in 37% and 45% inhibition of *P. berghei* respectively, while employing the compound with chloroquine in combination form with ratios of 90/10 and 70/30 (chloroquine/chitosan) had a considerable potentiation including 71.58% and 83.85% inhibition effectiveness against *P. berghei*. Moreover, 20 mg/L (CCM) concentration of chitosan alone could eliminate 69.55% of *P. falciparum* in culture medium while in combination with chloroquine in ratios of 90/10 (chloroquine/chitosan) had considerable potentiation including 79.14% inhibition effectiveness. Mean survival time of those mice received combination therapy in ratios of 90/10 and 70/30 (chloroquine/chitosan) was longer than those took up mono therapy of either chloroquine or chitosan based on their ED50^s doses.

Conclusion: Interaction between chloroquine and chitosan showed considerable potentiation in combination form against either *P. berghei* or *P. falciparum* using in vivo and in vitro tests respectively. Meanwhile, interaction between the above mentioned agents resulted in a notable survival time for those *P. berghei*-infected mice treated with the combination.



Introduction

Malaria infection is one of the most important parasitic diseases in tropical and subtropical areas of the world (1). According to the annual report of WHO about 219 million cases were recorded as new cases with 435000 cases of death in the world in 2017 (2). Combating malaria infection depends on some factors including accurate treatment of the diseases. Spreading drug resistance in many strains of *Plasmodium falciparum* in the most malarious areas (3) and recent years in some strains of *P. vivax* (4) is an obstacle in the pathway of combating malaria diseases. Besides the mentioned problem a number of limitations such as side effects of antimalarial drugs for pregnant women, people with G6PD deficiency and those with nephrotic syndrome lay some barriers in the way of proper control of malaria. To overcome the stated limitations efforts for obtaining novel and effective compounds are recommended by some of malaria policy makers. The use of natural resources particularly in combination form for treatment of malaria infection has been greatly interested by relevant researchers. The use of antimalarial drugs in combination form with the number of compounds may potentiate each other's effectiveness and will delay development of drug resistance in plasmodia parasites and this approach has been used with some success. Although interaction between various classes of antimalarial drugs have been approved for many years, there are not extensive knowledge about interaction between antimalarial and non-antimalarial drugs. Mazhari et al. in an interaction assessment between *Heracleum persicum* (HP) and chloroquine (CQ) found a synergistic interaction between the compounds in ratio of 40% CQ+60% HP, however, either additive or antagonistic effects in the other ratios against chloroquine-sensitive strain of *P. berghei* (5). In another

study, an antagonistic interaction was found between *Artemisia aucheri* and chloroquine against chloroquine-sensitive *P. berghei* (6). More recently a number of compounds are capable to reverse chloroquine (CQ) resistance in human plasmodia (7,8).

Chitosan (C₆H₁₁NO₄) (CS), a modified carbohydrate polymer of amino acetyl of chitin, is used as a basic substance in some medicines, food industries, textile industries and biochemical products (9). Chitosan is the most abundant natural polysaccharide after Cellulose. The substance is produced as a result of connecting glucosamine monomers together and has no environmental harmful effects. Easy availability and inexpensive cost are the others advantages of the chemical. Chitosan is available in solution, powder, flake and fiber forms.

Chitosan has been extracted from about 300 resources such as plants, aquatic invertebrates and insects. For the present study shrimps and crabs were the main utilized sources of the chemical. According to our knowledge exposing *Plasmodium* species to extract of chitosan rarely have been considered in the field of malaria (10).

The present study was conducted to investigate the effect of chitosan alone and in combination with chloroquine on *P. berghei* and *P. falciparum* using in vivo and in vitro tests respectively. Chloroquine was used due to its some advantages such as inexpensiveness, sharp blood schizontocidal virtue against the most human Plasmodia species and to have minimum side effects for malaria patients. Meanwhile, reversing resistance in chloroquine resistant strains of *P. falciparum* in combination form may excite malaria policy makers to consider utilizing this beneficial antimalarial drug again.

Materials and Methods

This study was conducted in the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran in 2018 based on two methods including in vivo and in vitro tests as follows:

In vivo test

Preparation of chloroquine and chitosan solutions

One milligram (mg) of Chloroquine diphosphate (CQ) (Sigma chemical co.) was dissolved in 10 ml distilled water as stock solution for preparing successive concentrations. Moreover, 0.8 mg of Chitosan (CS) (Sigma - Aldrich co.) was dissolved in a composition of 9.9 (mL) normal saline with 0.1 mL Acetic acid as stock solution for making up next concentrations.

Determination of 50% effective dose (ED50) of chloroquine and chitosan

Fifty male white BALB/C mice with weight of 20 gr were kept in breeding cages and room temperature. The mice were fed normally with relevant food and tap water and divided into ten groups of five mice in each group. The mice were infected intraperitoneal with CQ-sensitive NICD strain of *P. berghei* by 0.2 ml of 106 *P. berghei* -infected RBCs/ μ l. To expose the mice to chloroquine and chitosan, Peter's method (11) with some modifications was employed. For determining ED50s of chloroquine and chitosan the mice were exposed to concentrations of 1, 3, 10 and 20 mg/kg and 10, 20, 40 and 80 mg/kg of the substances respectively two hours after parasitized RBCS injection. Although mice were infected with parasite via intra peritoneum injection, the treatment was exerted via subcutaneous injections. Treatment was continued once daily and lasted up to four days. In each of days 4, 7, 14, 21 and 28 a thin blood smear was prepared from tail of the mice. The smears were stained with Giemsa stain and then parasitemia per-

cent was calculated against 10,000 RBCs using light microscope with magnification of 1000.

The results were analyzed using SPSS software (Chicago, IL, USA).

Combination of chloroquine and chitosan

Combination effectiveness of chloroquine and chitosan was determined based on fixed ratios technique (12). Forty male white BALB/C mice, dividing into 8 groups with 5 mice in each group, were specified for this part of tests. The mice were infected with CQ-sensitive *P. berghei* as mentioned above and then were treated subcutaneously with ratios of 0/100, 10/90, 30/70, 50/50, 70/30, 90/10 and 100/0 CQ and CS respectively up to 4 days. Parasitemia determination was conducted as mentioned for ED50^s. Interaction rate between chloroquine and chitosan was computed as follows; The ED50^s concentration of the agents were plotted on two ordinates and these two values joined with a straight line. Results of the fixed ratios were plotted as points in between the ordinates. If the resulting fixed ratio points lay above the line potentiation (synergism) was indicated, namely the two agents influenced positively each other's activity. In contrast, if the points dropped below the line antagonism was indicated, namely the agents interfered with one another's action. Eventually, if the two agents had no effect other's action then the points would lie on or about the line, indicating an additive effect.

In vitro test

Cultivation of P. falciparum

A chloroquine - sensitive 3D7 strain of Plasmodium falciparum was cultivated according to Trager and Jensen method (11) with some modifications in this study. Briefly, a preliminary culture medium (PCM) including 1000 ml RPMI 1640 (Gibco), 0.056 gr of hypoxanthine in 10 ml distilled water, 0.05 grs Gentamycin in 5 cc distilled water and 20 μ l of 5 molar NaOH were mixed and stirred for four hours. The final solution was filtered and

dispensed into some 250 ml bottles. At the time of testing the PCM was enriched with 15% AB+ human serum to be made up complete culture medium (CCM). Meanwhile, all of the mentioned processes were conducted under the aseptic situation.

Preparation of chloroquine and chitosan Solutions

The solutions were made up with 1 mg chloroquine in 1 ml distilled water as stock solution. One microliter (μ l) of the solution was mixed with 999 μ l CCM (1 mg/l CCM) for making up successive concentrations. Moreover, 0.8 mg chitosan was dissolved in composition of 9.9 ml normal saline and 100 μ l acetic acid as stock solution for preparing next concentrations.

Determination of 50% inhibitory concentration (IC50) of chloroquine and chitosan

A chloroquine - sensitive strain *P. falciparum* 3D7 was cultivated according to Trager and Jensen method. For determining IC50 of chloroquine and chitosan, concentration of 1, 2, 4

and 6 mg/l and 1.25, 2.5, 5, 10, 15 and 20 mg/l respectively were allocated into wells of microtiter plates. Three wells in each row were set up for each concentration and addition to the test rows two others also were specified for background including non-infected RBC^s and untreated positive control. The test wells received 10 μ l of parasite-infected blood with 1% parasitemia and 90 μ l of relevant concentration of the agents. Positive control wells were charged with 10 μ l of parasite-infected blood plus 90 μ l of normal CCM. Microtiter plates were placed in a candle jar and kept in an incubator with 37 °C temperature for 24 hours. At the end of incubation period the jar was taken out from incubator and then a thin blood smear was made from each of test and control wells. Parasites percentage were calculated against 10,000 infected and non-infected RBC^s. Parasitemia and inhibition percent were calculated according to the following equations. Concentration of fifty percent inhibition was computed by using a semi-log sheet.

$$\text{Parasitemia \%} = \frac{\text{number of parasites in the test}}{\text{number of parasites in control}} \times 100$$

$$\text{Inhibition \%} = \frac{\text{parasitemia in control} - \text{parasitemia in the test}}{\text{number of parasitemia in control}} \times 100$$

Combination of chloroquine and chitosan

A fixed ratio method (as mentioned for in vivo test) was used for combination of chloroquine and chitosan with ratios of 0/100, 10/90, 30/70, 50/50, 70/30, 90/10 and 100/0 based on IC50 of the agents. Process of the in vitro tests for combination was conducted as determining IC50^s, unless the individual concentrations were replaced with the ratios. Moreover, plotting the results and computing potentiation, antagonism and additive effects between the agents were conducted as the same of in vivo tests.

Survival time

Survival time was calculated based on number of days that the treated mice survived.

Ethics approval

Implementation of this study was permitted by Research Ethic Committee of Tehran University of Medical Sciences (REC .TUMS) under the CodeNo.IR.TUMS.VCR.REC.1396.3022.

Results

In vivo tests

Administering 1 and 3 mg/kg of chloroquine to *P. berghei*-infected mice up to four consecutive days resulted in 39% and 85.57% inhibition of the parasites on D4 respectively and two higher concentrations i.e.10 and 20 mg/kg removed completely the parasites at the same time, ED50 was computed as 1.5 mg/kg.

In this manner, administering 10 and 20 mg/kg of chitosan to the infected mice up to four successive days resulted in 37% and 45% inhibition of *P. berghei* on D4 respectively. The two higher concentrations i.e.40 and 80 mg/kg had not any effect on growth of the parasite. Based on the above results ED50 was replaced with ED45 for combination tests.

Results of interaction between chloroquine and chitosan against *P. berghei* pointed that ratios of 90/10 and 70/30 of CQ and CS respectively had a considerable potentiation against *P. berghei* (Table 1 & Fig. 1).

Table 1: Effectiveness of different ratios of chloroquine and chitosan on *P. berghei* in infected mice

Groups	Ratio of the drugs	Percentage of inhibitory values ($\bar{x} \pm SD$)
1	100% CQ	45.96±10.17
2	90%CQ+10%CS	71.58±4.01
3	70%CQ+30%CS	83.85±8.16
4	50%CQ+50%CS	5.52±5.67
5	30%CQ+70%CS	11.74±10.55
6	10%CQ+90%CS	9.08±7.13
7	100%CS	39.76±24.96

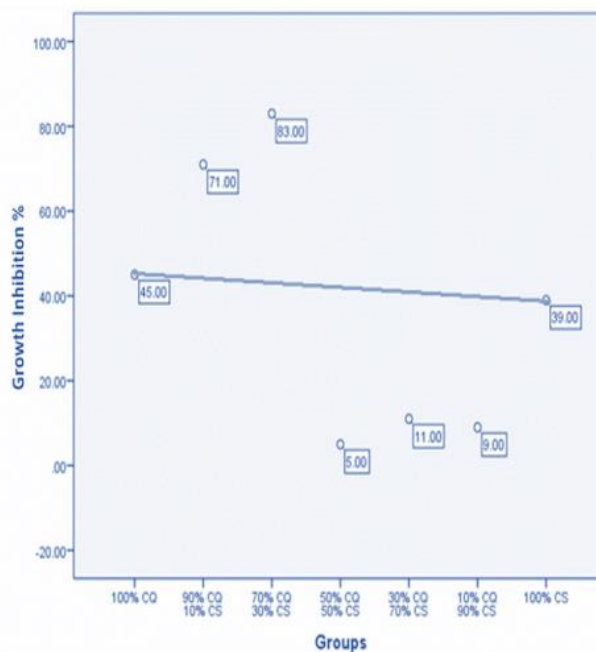


Fig. 1: Interaction between chloroquine and chitosan on *P. berghei* in infected mice

Survival time of the mice

Mean survival time of those mice received combination therapy in ratios of 90/10 and 70/30 of chloroquine and chitosan respective-

ly was longer than those took up mono therapy either CQ or CS based on their ED50^s doses ($P < 0.05$) (Fig. 2).

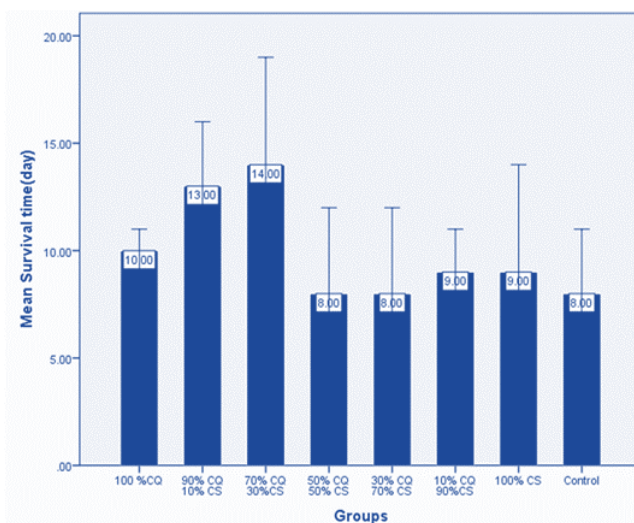


Fig. 2: Mean survival times bare of *P. berghei* infected mice treated with combination of CQ & CS

In vitro tests

Results of exposing 3D7 strain of *P. falciparum* to different concentrations of chloroquine

and chitosan using In vitro tests for 24 h have been shown in Tables 2 & 3.

Table 2: Effectiveness of different concentrations of chloroquine on 3D7 strain of *P. falciparum*

Rows	Concentration of the drug	Percentage inhibitory growth ($\bar{x} \pm SD$)
1	1 mg/l CCM	43.3 ± 10.2
2	2 mg/l CCM	76.1 ± 15.4
3	4 mg/l CCM	93.5 ± 10.8
4	6 mg/l CCM	93.95 ± 8.2

Table 3: Effectiveness of different concentrations of chitosan on 3D7 strain of *P. falciparum*

Rows	Concentration of the drug	Percentage inhibitory growth ($\bar{x} \pm SD$)
1	1.25 mg/l CCM	22.65 ± 10.6
2	2.5 mg/l CCM	26.14 ± 7.4
3	5 mg/l CCM	40.98 ± 1.2
4	10 mg/l CCM	39.59 ± 0.58
5	15 mg/l CCM	57.55 ± 10.6
6	20 mg/l CCM	69.55 ± 1.9

According to the results chitosan in concentration of 20 mg/liter (CCM) could eliminate 69.55% of *P. falciparum* in culture medium while 6mg/liter of CQ could eliminate 93.95% of *P. falciparum*. Based on our results, IC50^s of CQ and CS are 1.4 and 5 mg/liter respectively.

Results of interaction between chloroquine and chitosan against 3D7 strain of *P. falciparum* showed that ratios of 90/10 (CQ/CS) had a considerable potentiation including 79.14% inhibition effectiveness against the parasite (Table 4 and Fig. 3).

Table 4: Effectiveness of different ratios of chloroquine and chitosan on 3D7 strain of *P. falciparum*

Rows	Concentration of the drug	Percentage inhibitory growth ($\bar{x} \pm SD$)
1	100% CQ	54.12±15.86
2	90%CQ+10%CS	79.14±6.9
3	70%CQ+30%CS	57.97±24.67
4	50%CQ+50%CS	65.91±13.2
5	30%CQ+70%CS	67.49±9.05
6	10%CQ+90%CS	69.03±6.19
7	100%CS	40.0±10.0

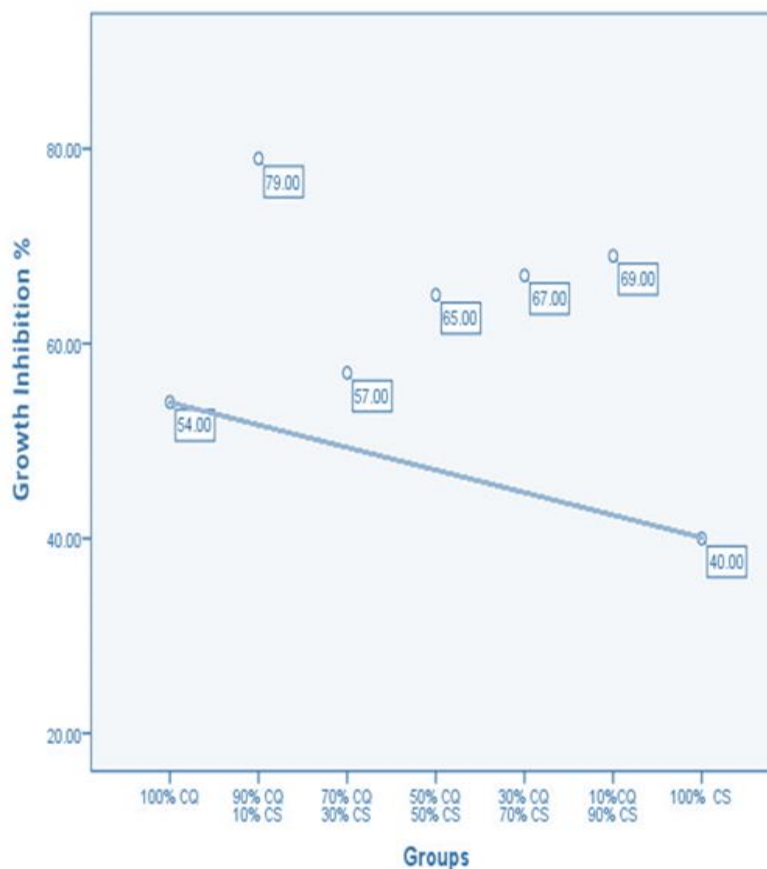


Fig. 3: Interaction between chloroquine and chitosan on 3D7 strain of *P. falciparum*

Discussion

Although chloroquine, an effective antimalarial drug, had been used for many years against *P. falciparum* and still is used against other species of human malaria, nowadays spreading chloroquine resistant strains of *P. falciparum* in the world persuades malaria policy makers to search novel antimalarial drugs particularly among the natural products. Chitosan is a derivative of glucan with recurred units of chitin. This compound has considerable effects on some fungi and bacteria such as pseudomonas. Chitosan is abundantly found in outer skeleton of Arthropods such as shrimps, crabs, insects as well as some plants. The agent is gentle with environment and cost effective.

Our study showed that chitosan can effect individually or in combination with chloroquine on *P. berghei* and *P. falciparum* to eliminate the parasites from mice and parasite cultures respectively. Teimori and colleagues showed that chitosan with 20 mg/kg (of mouse body weight) could eliminate 49.20% of *P. berghei* in fourth day of four days treatment in mice (10) which is in agreement with the result of our study. In our in vitro study chitosan could inhibit 69.55% growth of 3D7 strain of *P. falciparum* in continues culture. Such difference can be explained due to differentiating either in species or presence of some interfering factors in in vivo tests.

Although chitosan alone could not eliminate *P. berghei* completely at the end of treatment duration in mice, showed a considerable result in combination with chloroquine against the parasite with 83.85% inhibition in ratios of 70/30 (CQ/CS).

Interaction between chloroquine and some medicinal herbals against chloroquine-sensitive *P. berghei* resulted in additive and antagonistic results (12,13), but results of this study showed a potentiation between chloroquine and chitosan against both chloroquine-

sensitive *P. berghei* and *P. falciparum*. Moreover, survival time of those mice treated with combination of chloroquine and chitosan particularly in ratios of 90/10 and 70/30 (CQ/CS) confirms results of positive interaction between those compounds.

Comparing between those results obtained from in vivo and in vitro tests with different species of plasmodium particularly in ratios of 90/10 (CQ /CS) hints that interaction between chloroquine and chitosan can potentiate each other's effectiveness on both rodent and human plasmodia.

According to our knowledge since this study is the first consideration about interaction between chloroquine and chitosan against *P. berghei* and *P. falciparum*, so there is not any more data we can discuss about that. Indeed, more studies are needed to consider different aspects of interaction between chloroquine and chitosan against different species of rodent and human plasmodia.

Ratios of 50/50, 30/70 and 10/90 of chloroquine and chitosan respectively resulted in antagonism between the agents accompanied by increasing *P. berghei* parasite multiplication in the mice. Conversely, when *P. falciparum* was exposed to the above mentioned ratios combination of chloroquine and chitosan affected on the growth and multiplication of the parasite and resulted in potentiation between the agents. Such differences between results of in vivo and in vitro tests may happen due to influence of some biological factors in the mice on the above combination to prevent the effectiveness of the agents on *P. berghei*.

Conclusion

Interaction between chloroquine and chitosan showed considerable potentiation in combination form against either *P. berghei* or *P. falciparum* using in vivo and in vitro tests respectively. Meanwhile, interaction between the above mentioned agents resulted in a notable

survival time for those *P. berghei*-infected mice treated with the combination.

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Conflict of interest

All the authors declare that they have no conflict of interest.

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