PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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Pakistan Journal of Nutrition 15 (8): 789-794, 2016
ISSN 1680-5194

Combination of Chloroquine and Lycopene for Combating Malaria: A Case Study in Mice (*Mus musculus*) Infected with *Plasmodium berghei*

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Abstract: *P. falciparum* has developed resistance to chloroquine throughout the world. The consumption of tomato (*Lycopersicum esculentum* Mill) lycopene is known to be able to increase resistance to *Plasmodium* parasites by slowing emergence and extending the survival rate. It is possible to develop tomato lycopene as a potential anti-parasite drug. Forty-eight Swiss albino mice were divided into 4 groups and infected by injecting 0.2 ml of 1 x 10^7 erythrocytic stage parasites from the blood of infected mice into the peritoneal cavity. The treatments were administered for 14 days; the negative control (C-) group was administered the lycopene solvent (placebo) and the positive control group (C+) received the placebo in combination with 0.3125 mg/kg BW chloroquine. The T1 group was administered 10 mg/kg BW/day tomato lycopene and the T2 group received 10 mg/kg BW/day tomato lycopene and 0.3125 mg/kg BW chloroquine. The administration of 10 mg/kg BW/day tomato lycopene is able to increase the levels of the cytokines IL-4 (4.196 pg/ml), IL-12 (4.050 pg/ml) and IFN-γ (10.061 pg/ml) and improves T cell proliferation (0.271 OD), whereas the combination of lycopene and chloroquine was able to significantly increase the phagocytic index (18.407%). However, chloroquine was not able to increase the NO levels (0.459 μm). Tomato and chloroquine were also able to delay parasitemia until 6 days after infection and increased survival to 22.33 days.

Key words: Chloroquine, malaria, tomato lycopene

INTRODUCTION

Chloroquine or 4-aminoquinoline is widely used to combat malaria resulting from *Plasmodium vivax*, *P. ovale* or *P. falciparum* infection (Amir and Zunilda, 2007). Chloroquine-resistant *P. falciparum* was reported in 1973 in East Kalimantan and spread to 27 other Indonesian provinces (Tjitra, 2004). The proportion of chloroquine-resistant strains in high case incident (HCI) areas in Central Java achieved 28.57% and increased to 62.79% in 2007 (Tuti *et al*., 2007).

Chloroquine-resistant *P. falciparum* has been identified around the world, but chloroquine is still used in several countries (Winstanley *et al*., 2004). The countries that currently recommend chloroquine as chemoprophylaxis for travelers include China and countries located in Central and South America (Panama, Haiti, Dominican Republic and Argentina) and the Middle East (Syria, Jordan and Iraq). Chloroquine resistance is caused by (1) drug factors, such as the use of drugs in sub-therapeutic doses; (2) parasitic factors, such as genetic mutations, environmental factors and vectors and (3) human factors, such as incompatible immune responses.

One of the efforts to improve resistance to *P. falciparum* by improving the immune response is the use of tomato (*Lycopersicum esculentum* Mill). Tomato contains more lycopene than guava, red grape, watermelon and other red fruits (Bub *et al*., 2000; Arab and Steck, 2000). The consumption of tomato lycopene is able to increase interleukin (IL)-12 and IL-4 levels, induce interferon-γ (IFN-γ) production and enhance macrophages. It is possible that the incompatibility of the anti-malarial agent chloroquine is enhanced when it is combined with tomato lycopene. This study aimed to understand the effect of tomato lycopene use in terms of increasing the effectiveness of chloroquine.

MATERIALS AND METHODS

Ethical clearance was obtained from the Health Research Ethics Committee, Faculty of Medicine, Universitas Diponegoro (UNDIP) Dr. Kariadi Hospital, Semarang, Indonesia. This study is an *in vivo* laboratory experiment using forty-eight 6 to 8 week old male Swiss albino mice (*Mus musculus*) that were obtained from the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada (UGM) Yogyakarta. The mice were reared intensively at the Laboratory of Parasitology, Faculty of Medicine, UGM. All mice weighed 29-30 g and the weights were homogeneous (p = 0.987).

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Swiss albino mice were infected by injecting 0.2 ml of 1 x 10^7 erythrocytic stage parasites from the contaminated blood of infected mice into the peritoneal cavity. Forty-eight mice were divided into 4 groups. The treatments were conducted for 14 days. The negative control (C-) group was administered the lycopene solvent (placebo), whereas the positive control group (C+) received the placebo in combination with 0.3125 mg/kg chloroquine. The T1 group was administered 10 mg/kg BW/day tomato lycopene and the T2 group received 10 mg/kg BW/day tomato lycopene and 0.3125 mg/kg BW chloroquine.

Six mice from each group were randomly selected and sacrificed to measure T cell proliferation; the phagocytic index; titers of IL-12, IL-4 and IFN-γ; NO levels; parasitemia and the prepatent period. Then, six mice per group were left alive until death to measure viability. Research reagent kits, such as ELISA and Giemsa and Griess reagents, were obtained from Bender Medical Systems (Jakarta, Indonesia).

IL-12, NO levels and phagocytic index: The IL-12 and NO levels were measured in the supernatants of centrifuged macrophage cultures. The IL-12 levels were measured using a mouse ELISA kit for IL-12, whereas the NO levels were measured using a modified Griess method. Absorption was monitored using a microplate reader with a wavelength of λ = 450 nm for IL-12 and λ = 450 nm for NO. The phagocytic index was calculated by measuring the amount of Latex beads that were phagocytosed by the macrophage cultures.

IL-4 and IFN-γ levels and T cell proliferation: The IL-4 and IFN-γ levels were measured in the supernatants of centrifuged mouse spleen lymphocyte cultures. Then, both the IL-4 and IFN-γ levels were measured using the mouse ELISA kit for IL-4 and IFN-γ. The respective IL-4 and IFN-γ levels were obtained on a microplate reader by reading the absorbance of the supernatant at 450 nm. The number of cultured mouse spleen lymphocytes was counted to measure T cell proliferation by dimethylthiazol-thiazolyl Blue Diphenyltetrazolium bromide (MTT) assay and the results were determined with an ELISA.

Parasitemia levels, prepatent period and viability: Parasitemia levels were observed in a series of thin blood smears that were stained using Giemsa and then observed under a light microscope at 1000 X magnification. The specified prepatent period was observed from the beginning of the *P. berghei* infection until the discovery of merozoites in the peripheral blood. Mouse viability was determined by the ability of the animal to survive after infection with *P. berghei*, which was calculated by determining the number of days from infection until death.

RESULTS

The mice used in this study had similar average weights that were not significantly different and were homogeneous ($p \geq 0.05$). The treatment of the mice during the research process resulted in significant differences in the measured parameters.

IL-12 levels, NO levels and phagocytic index: The macrophage phagocytic index measures the ability of macrophages to eliminate pathogens in the body. The phagocytic index of the T2 group increased to 18.407% and was significantly different from the results of the other treatments. The administration of lycopene to the T1 group increased the phagocytic index to 5.179%, but it was not significantly different from the C+ group (6.356%).

The phagocytic index of the T2 group was high and was accompanied by increased IL-12 and NO levels, which were significantly different from those of the control groups (Table 1). Tomato lycopene was able to increase the IL-12 titer level in the T1 group to 4.050 pg/ml, which was significantly different from that of the C-, C+ and T2 groups. The IL-12 titer level in the T2 group increased to 1.338 pg/ml and was significantly different from that of the C- group at 1.080 pg/ml and that of the C+ group at 0.687 pg/ml.

The NO level of the T2 group was equal to 0.459 μM and was similar and not significantly different from the NO level of the T1 group, which was 0.452 μM. However, there were significant differences compared with the control groups, whose NO levels reached 0.334 μM for the C+ group and 0.289 μM for the C- group. NO has an important role in the phagocytic mechanism of macrophages. This result shows that the administration of chloroquine was not able to increase NO production and did not have a significant impact on the increased IL-12 titer levels in the *P. berghei*-infected mice. However, chloroquine was able to increase the phagocytic index.

T cell proliferation and IL-4 and IFN-γ levels: T cell proliferation in mice that were administered tomato lycopene and chloroquine (T2) was as high as 0.271 OD. This value was higher and significantly different from the 0.136 OD of the T1 group, which was only administered tomato lycopene, the 0.121 OD of the C+ group, which was administered tomato lycopene solvent and chloroquine and the 0.116 OD of the C-group (Table 2).

Though the titer level of IL-4 in group T2 increased to 2.745 pg/ml, it was lower than the level in the T1 group, which was 4.196 pg/ml and was not significantly different from the level in the C-group, which was 2.364 pg/ml. The result shows that the administration of chloroquine to mice infected by *Plasmodium berghei* did not result in increased titer levels of IL-4. Moreover, the treatment used in the T2 group did not result in higher IFN-γ levels than in the T1 group.

Table 1: Results of the LSD test of the effects of tomato and chloroquine on the IL-12 levels, NO levels and phagocytic index*

Groups (n = 6)	Phagocytic index	IL-12 levels (pg/ml)	NO levels (μM)
C- (Placebo)	0.493[a]	1.080[a]	0.289[a]
C+ (Chloroquine)	6.356[b]	0.687[a]	0.334[a]
T1 (Lycopene)	5.179[b]	4.050[b]	0.452[b]
T2 (Lycopene + chloroquine)	18.407[c]	1.338[c]	0.459[b]

p≤0.05. Different letters (a-d) indicate a significant difference among groups
*The values indicate statistical significance at or above a 95% confidence level using the LSD test

Table 2: Results of the LSD test of the effects of the tomato lycopene and chloroquine treatments on T cell proliferation, IL-4 levels and IFN-γ levels*

Groups (n = 6)	T cell proliferation (OD)	IL-4 levels (pg/ml)	IFN-γ levels (pg/ml)
C- (Placebo)	0.116[a]	2.364[a]	6.314[a]
C+ (Chloroquine)	0.121[ab]	1.944[a]	5.852[a]
T1 (Lycopene)	0.136[b]	4.196[b]	10.061[b]
T2 (Lycopene + chloroquine)	0.271[c]	2.745[ac]	7.193[c]

p≤0.05. Different letters (a-d) indicate a significant difference among groups
*The values indicate statistical significance at or above a 95% confidence level using the LSD test

Table 3: Results of the LSD test of the parasitemia levels and *P. berghei*-infected mice viability*

	---------- Parasitemia levels by day ----------					
Groups (n = 6)	2nd	4th	6th	8th	Prepatent period (days)	Viability (days)
C- (Placebo)	1.083[a]	8.900[a]	14.017[a]	20.450[a]	2	9.5[a]
C+ (Chloroquine)	0.100[b]	0.967[b]	4.650[b]	8.650[b]	2	18.83[b]
T1 (Lycopene)	0[b]	0[c]	2.317[c]	6.817[c]	6	15.5[c]
T2 (Lycopene + chloroquine)	0[b]	0[c]	0[d]	0.117[d]	8	22.33[d]

p≥0.05. Different letters (a-d) indicate a significant difference among groups
*The values indicate statistical significance at or above a 95% confidence level using the LSD test

The IFN-γ level in the T2 group was 7.193 pg/ml. This value was lower than the IFN-γ level in the T1 group, which was equal to 10.061 pg/ml, but was higher than that of the C+ group, which was equal 5.852 pg/ml. This result indicates that the administration of tomato lycopene could significantly increase cytokine production compared to chloroquine.

Parasitemia levels and prepatent period: A significant difference in the parasitemia level was observed between the T2 group and with the C-, C+ and T1 groups. On the 8th day, the parasitemia level of the T2 group (0.117%) was less than that of the T1 (6.817%) and C+ (8.650%) groups. Decreased levels of parasitemia are caused by an increase in the immune response, which was modulated by tomato lycopene.

Microscopically, the effects of tomato lycopene appeared in *P. berghei*-infected red blood cells during the infection period until the mice were sacrificed. The parasitemia levels in each group were different on each day. The *P. berghei* phases that appeared in the blood streams of the mice were trophozoites and schizonts.

Hours after infection, the blood samples showed a few red blood cells infected with *P. berghei* in ring form. Then, on the 2nd day, the erythrocytes were infected with trophozoites. On the 4th day after infection, the trophozoites evolved into early schizonts; at this phase, purple-pigmented schizont clumps with Maurer's clefts were observed. On the 6th day after infection, the parasites evolved into mature schizonts. On the 8th day, the erythrocyte cell membrane was lysed and the parasite entered the blood vessel (Fig. 1). These parasite will be phagocytosed by macrophages, but several parasites that are not phagocytosed will infect new erythrocytes.

Viability: Improved function and activity of the immune response, which is modulated by tomato lycopene, had a greater effect on the ability of the infected mice to survive. The mice in the T2 group that were administered tomato lycopene and chloroquine were able to survive 22.33 days, which was longer than the survival of the T1 group at 15.5 days and the C+ group at 18.83 days.

DISCUSSION

Immune responses to malaria are initiated by interactions between parasites and the major histocompatibility complex II (MHC-II) on the macrophage cell surface. The interaction activates macrophages to synthesize cytokines, such as IL-12, which are able to induce lymphocyte proliferation. In addition, macrophage activation increases NO production to destroy the infecting *Plasmodium* (Percario *et al*., 2012).

Chloroquine prevents the digestion of hemoglobin, which is necessary for the parasite life cycle, thereby reducing the amino acid supply. The administration of chloroquine as an anti-malarial agent plays an important role in preventing the proliferation of *P. berghei* in red blood cells. Chloroquine will form an uncharged molecule at neutral

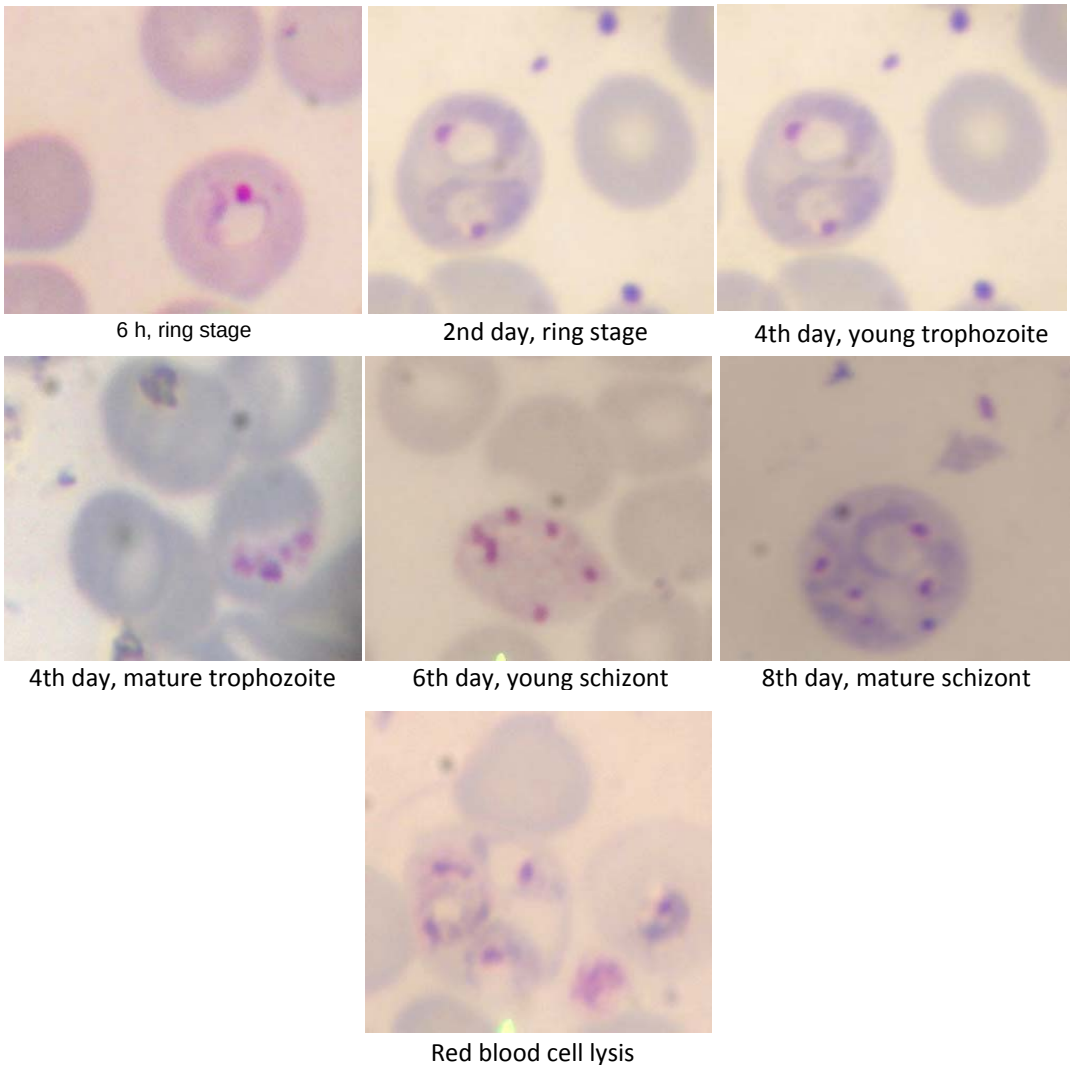

Fig. 1: *P. berghei* erythrocyte stage in mouse red blood cells up to 8 days after infection

pH; thus, it easily diffuses into the *P. berghei* lysosomes. The acidic pH of the lysosome changes the neutral chloroquine into a protonated form that causes impermeable molecules to pass through the membranes (Tilley *et al*., 2011).

Chloroquine resistance in malaria is caused by mutations in *P. falciparum* genes, such as the chloroquine resistance transporter (*pfcrt*) gene on chromosome 7 (Laufer *et al*., 2010). The *pfcrt* gene mutation is manifested as a survival adaptation toward long-term chloroquine consumption. The *pfcrt* protein is able to neutralize chloroquine in the *Plasmodium* body by re-pumping chloroquine out of the *Plasmodium* body (Boddey and Cowman, 2013).

P. berghei requires amino acids for the growth and reproduction phases (from trophozoite to schizont). The need is met by digesting hemoglobin from red blood cells, which releases large quantities of iron ions (Fe) into the blood circulation, triggering oxidative stress and increasing the production of reactive oxygen and nitrogen species (ROS and RNS) (Kumar and Bandyopadhyay, 2005). The ROS and RNS in the body are neutralized by endogenous and exogenous antioxidants. Carotenoids in the body are capable of scavenging oxidants and reducing the negative impacts, giving the body the ability to heal faster and live longer (Table 3).

In addition, the administration of 10 mg/kg BW tomato lycopene was shown to increase the IL-4, IFN-γ and NO titer levels and macrophage activity (Table 1 and 2). The increased cytokine production was caused by immune cell activation, such as macrophages, lymphocytes and natural killer (NK) cells. Meanwhile, the administration of chloroquine did not affect cytokine production or macrophage activity and was not involved in the specific immune response (Table 1 and 2). Immune responses, particularly T cell and macrophage activation, are affected by the antioxidant status (Raverdeau and Mills, 2014)

Lycopene is a carotenoid that can produce intermediates and precursors of vitamin A (Perveen *et al*., 2015). Carotenoids and vitamin A are converted into retinoic acid (RA) (Lobo *et al*., 2010). Retinoic acid improves T cell proliferation and cytokine production after binding with Retinoic Acid Receptor/Retinoid X-Receptor (RAR/RXR) (Zapata-Gonzalez *et al*., 2007). RA-RAR/RXR stimulate the transcription of cytokine mRNAs and increase the synthesis of cytokine proteins such as IL-2, IL-4, IL-12 and IFN-γ (Hill *et al*., 2008; Nolting *et al*., 2009; Takeuchi *et al*., 2013).

Based on research findings, the amount of IL-12 production was consistent with the number of activated T cells (Table 1 and 2). IL-12 activates naive T cells into T reg and/or Th1 cells (Takeuchi *et al*., 2013). Activated T cells produce IL-4 and IFN-γ to stimulate macrophages, NKs and dendritic cells (DC) to perform antigen phagocytosis. According to Abbas and Lichtman, macrophages produce IL-12 in response to sporozoites (Abbas and Lichtman, 2012).

IL-12 production stimulates T cells to produce IFN-γ, which then activates macrophages to phagocytose sporozoites. The roles of the IFN-γ produced by Th-1 cells also include inhibiting the activity of Th-2 cells and suppressing humoral immune responses.

The phagocytic activity of macrophages was observed as an increase in NO production (Table 1). NO production is influenced by IFN-γ, which activates the transcription of genes that encode phagocyte oxidase. Phagocyte oxidase is an intermediate enzyme that produces reactive oxygen species and inducible nitric oxide synthase (iNOS). NO kills the parasite through two mechanisms. First, NO inactivates the electron transport chain in the mitochondria. Second, NO reacts with superoxide anions to form peroxides, which are broken after protonation compounds of highly toxic hydroxyl radicals are formed.

The administration of low doses of chloroquine and lycopene is able to reduce the number of parasites and delay their emergence. This treatment increased the immune responses in mice and prolonged life longer than the administration of chloroquine or lycopene alone. In addition, the low levels of parasitemia showed that the administration of chloroquine and 10 μM lycopene was able to eliminate the intracellular parasites.

Conclusions: The administration of 10 mg/kg BW/day tomato lycopene with low doses of chloroquine in Swiss albino mice infected with *Plasmodium berghei* increases the potency of chloroquine as an anti-malarial drug by increasing viability, decreasing parasitemia and increasing prepatent periods, T cell proliferation, IFN-γ titer levels, NO levels and the macrophage phagocytic index. It is also able to reduce the parasitemia levels and increase the prepatent period and vitality. However, chloroquine alone is not able to increase the cytokine levels.

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