

## The effect of vitamin C on morphology and histology of liver and spleen of *Plasmodium* - infected mice

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### Abstract

The purpose of this study was to evaluate effects of vitamin C on both liver and spleen morphology and histology after vitamin C consumption in *Plasmodium*-infected mice. *Plasmodium yoelii* 17X (lethal) strain was murine malaria we generated and ICR mice were used as a model. Mice were randomly divided into 3 groups; normal control (C), parasite infection only (P-only) and vitamin C consumption after parasite infection (P+VitC). After the mice died, livers and spleens were removed. Colors were noticed, weights and sizes were measured. Hematoxylin and eosin technique was performed and photos were taken. The colors of both livers and spleens were found similar between P-only and P+VitC mice. P+VitC mice livers and spleens showed no significant difference in weight if compared with C mice. P+VitC mice livers are also showed no significant difference in size if compared with C mice whereas P+VitC mice showed significant difference in bigger size than C mice in spleen. For histology, liver of C mice showed normal appearance whereas abnormal appearances and the finding of haemozoin were found in P-only and P-VitC livers. For spleen, normal white and red pulps were seen in P+VitC mice similar to the observation on C mice. However, hemozoin were still found in splenic sinusoids of P+VitC mice. In conclusion, taken the results together, it is likely that VitC alone does not completely help reducing the damages in both morphology and histology of liver and spleen. However, adding VitC as the supplement to other candidate drugs should be considered.

### Keywords

Malaria  
*Plasmodium yoelii*  
Vitamin C  
herbal medicine  
spleen  
liver

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### Introduction

Malaria is one of the most important public health problems caused by the protozoa of genus *Plasmodium* that can transmit through the bite of mosquitoes. About 216 million cases were detected and 655,000 cases which died in 2010 were reported by World Health Organization in 2011. Currently, chemotherapy is still the best choice for human to control malaria infection. Since there is no vaccine available for malaria, in addition, nowadays, there is the widespread of antimalarial drugs resistance, for instance, chloroquine, pyrimethamine and mefloquine resistances. Thus, novel safe, effective and accessible new treatments have to become the priority needs. Recently, herbal plants which are easily found in the local areas play a crucial role in treatments. They have been used for ages to cure lots of infectious diseases including malaria. Thus, it is likely that herbal plants might be the most convenient solution for being novel drug candidates because of their diversity and is

easily found in nature. Thailand is one of the tropical countries which malaria is endemic. In addition, Thailand has variety of plants which are widespread all over the country, easily found and cost cheap. However, there is less number of reports about Thai local herbal plants against the diseases. Therefore, investigating Thai local plants is needed. Since, the use of plants for malaria treatments is discussed, thus, this study attempts to investigate the new Thai local herbal plant if it may provide curing effect against malaria infection. Vitamin C or ascorbic acid which can extract from *Citrus* spp. is known as an antioxidant and strong immune moderator (Meydani and Beharka, 1998; Snow *et al.*, 2005). It has been documented that VitC is a contributor to fight free radicals damage in membrane lipid (Nmorsi *et al.*, 2007), in addition fighting against oxidative stress induced by malaria (Nmorsi *et al.*, 2007). During the erythrocytic stage of malaria parasite, malaria host-parasite interaction generates reactive oxygen species resulting both of them are under oxidative stress. The

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host cell itself alters erythrocyte membrane fluidity, most probably lipid components and protein – cross linkage (Muller, 2004). Parasite itself is also under oxidative stress since there are accumulations of  $Fe^{2+}$ ,  $Fe^{3+}$  in its food vacuole and also has been released upon haemoglobin digestion as haemozoin which must be detoxified (Muller, 2004). As described, VitC has its own properties especially, antioxidant. However, there is still limited information of the role of VitC on the outcome of malaria infection. Importantly, during malaria infection, liver is the organ in which the parasites reside in. Moreover, spleen, the lymphoid organ, also plays a crucial role in hematogenesis and detoxification during infection. Reports of unhealthy liver and spleen during malaria infection have been already known. Consuming VitC might help both organs recover to nearly normal appearances, possibly. Thus these two organs are included in this study to investigate the effects of VitC on the changes of morphology and histology of infected liver and spleen after consumption.

## Materials and Methods

### Experiment animals

Fifteen 6-week-old female ICR mice were purchased from National Laboratory Animal center, Mahidol University, Salaya, Nakhon Pathom, Thailand. These mice were kept for a week before starting the experiment. Mice were housed in standard diet and water with 12 hours light- dark cycles. This animal experimentation is approved by Ethical committee of Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

### Malaria parasite

*Plasmodium yoelii* 17X (lethal) – infected ICR stocks were maintained by serial passages and were stored in liquid nitrogen until used.

### Drug - Vitamin C (Ascorbic Acid)

Vitamin C powders were prepared freshly at the concentration of 100 mg/kg diluted with distilled water before using. This concentration was selected since it gave the good result in *in vitro* study (personal communication with Dr. Thidarut Boonmars).

### Experimental design

All ICR mice were randomly divided into 3 groups; each group contained 5 mice; group 1, 2 and 3; served as control (C), mice were inoculated by  $10^7$  *P. yoelii* 17XL only (P-only) and mice were inoculated by the  $10^7$  *P. yoelii* 17XL later VitC at the dose of 100 mg/kg was injected (P+VitC). Route of parasite inoculation was intra-peritoneal. Briefly,

on day 0, C-mice were fed with standard diet and water only whereas both P- only and P +VitC mice were inoculated with  $10^7$  *P. yoelii* 17XL. At day 1, only P+VitC mice were given 100 mg/kg of VitC. VitC was kept giving to this group of mice daily. Percent parasitemia was checked by Giemsa staining every day. All mice were followed daily until death. Immediately, livers and spleens were removed, photo taken, weights and sizes were measured and later processed to haematoxylin and eosin technique. One way-Anova was used for statistics analysis. Significantly difference was justified at  $p < 0.05$ .

## Results

### Morphology and histology of liver

Compared with C mice (Figure 1A), P-only and P+VitC livers showed similar color; brownish-black (Figures 1B-C), whereas C mice livers appeared reddish black in color (Figure 1A). For livers weights and sizes, there showed no significant difference in weight and size of P-only and P-VitC mice (Table 1). Investigating liver histology, C mice livers showed polygonal shape of hepatocytes with well arrangement of hepatic sinusoids. No malaria pigment or haemozoin was found (Figure 3A). Oppositely, in P-only and P+VitC livers, both of them displayed similar abnormalities in histology. Swelling hepatocytes and distended liver sinusoids were noticed. Haemozoin were found with Kupffer cells inside sinusoids (Figures 3B-C).

Table 1. Weights, lengths and widths of liver of each group of mice; n = 5 (mean  $\pm$  SD)

Group of mice	Weight (g)	Width (mm)	Length (mm)
C	2.16 $\pm$ 0.27	20.6 $\pm$ 0.24	21.0 $\pm$ 0.20
P-only	2.62 $\pm$ 0.23	22.0 $\pm$ 0.20	26.8 $\pm$ 0.68
P+VitC	2.18 $\pm$ 0.62	20.6 $\pm$ 0.18	26.2 $\pm$ 0.29



Figure 1. Colors of each group of mice livers; A: C mice, B: P-only mice, C: P+VitC mice

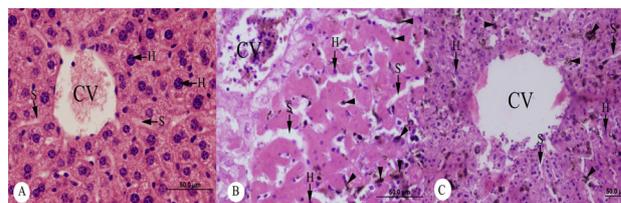


Figure 3. Histological appearances of mice livers (X20); A: C mice, B: P-only mice, C: P+VitC mice. CV: central vein, H: hepatocyte, S: sinusoid, arrow head: haemozoin

*Morphology and histology of spleen*

The colors of spleens were reddish-brown in C mice (Figures 2A) and brownish-black in P-only (Figure 2B) and P+VitC mice (Figure 2C). For spleen weight, compared with C spleens, no significant difference in weight was shown between the spleens of P-only and P+VitC groups. Interestingly, there showed significant difference in spleen sizes of P+VitC group if compared with C group (Table 2) For spleen histology, C and P+VitC spleens showed distinct white and red pulps with clearly marginal zones (Figures 4A, C) whereas fused white and red pulps with no marginal zone was only found in P-only (Figure 4B). Oppositely, P+VitC spleens showed similar appearances of white and red pulps as C spleens (Figure 4C). Deeply investigating into splenic sinusoids, lots of haemozoin which residing in both P-only and P+VitC spleens were shown (Figures 4B-C). Moreover, megakaryocytes were found in both P-only and P+VitC spleens (Figures 4B-C) as well.

Table 2. Weights, lengths and widths of spleen of each group of mice; n = 5 (mean ± SD)

Group of mice	Weight (g)	Width (mm)	Length (mm)
C	0.74±0.11	3.8±0.08	17.2±0.15
P-only	1.02±0.24	8.6±0.05*	29.2±0.08*
P+VitC	1.04±0.26	8.0±0.07*	31.0±0.49*



Figure 2. Colors of each group of mice spleens; A: C mice, B: P-only mice, C: P+VitC mice

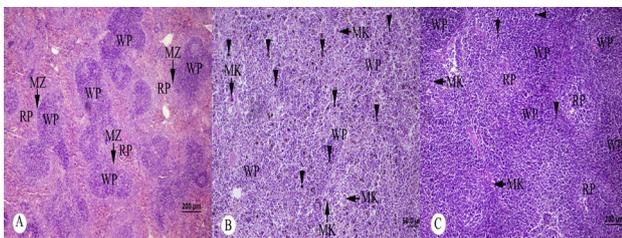


Figure 4. Histological appearances of mice spleens (X10); A: C mice, B: P-only mice, C: P+VitC mice. WP: white pulp, RP: red pulp, MZ: marginal zone, MK: megakaryocytes, arrow head: haemozoin

*Percent parasitemia*

No parasitemia was shown in C mice whereas in both P-only and P+VitC was shown continued increasing from day 1 until day 15. For P- only

mice, the maximum average percent parasitemia was found 97.70% on day 15. The maximum average total percent parasitemia of P + VitC mice was about 97.49% which was found on day 14. However, these two groups of mice finally dead at day 15. The average total percent parasitemia at the time of dead of P-only mice ranged from 95.25 - 97.70% whereas the P + VitC ranged from 77.91 - 96.23%. However there showed no significantly difference (Table 3).

Table 3. Average percent parasitemia in each group of mice, n = 5 (mean ± SD)

Day	Percent parasitemia in each group					
	Control (n=5)		P- only (n=5)		P+ VitC (n=5)	
	% parasitemia	No. of death	% parasitemia	No. of death	% parasitemia	No. of death
1	0	-	1.58±0.1	-	2.60±0.9	-
2	0	-	5.70±3.2	-	5.12±1.1	-
3	0	-	3.14±0.9	-	5.10±2.2	-
4	0	-	4.97±1.4	-	4.52±1.1	-
5	0	-	4.99±1.0	-	5.43±0.9	-
6	0	-	5.66±1.9	-	6.02±0.9	-
7	0	-	9.42±1.4	-	10.59±1.9	-
8	0	-	12.71±4.2	-	15.10±3.9	-
9	0	-	21.26±7.6	-	29.93±5.1	-
10	0	-	33.25±5.9	-	41.16±5.9	-
11	0	-	48.43±10.4	-	65.91±9.9	-
12	0	-	73.18±12.9	-	77.91±6.9	1
13	0	-	78.83±7.7	-	83.52±6.2	2
14	0	-	95.25±2.9	2	97.49±0	1
15	0	-	97.70±0.29	3	96.23±0	1

**Discussion**

The first part of this study was to investigate changes of liver and spleen by observing the color. This phenomenon may be due to the accumulation of haemozoin and the condition of anemia as supported by previous finding which occurred in *P. knowlesi* (Cox-Singh et al., 2010) and *P. chabaudi*, infection (Martiney et al., 2000). These changes were supported by the reduction of erythrocyte count and the increase of parasite in mice until they all died (Table 3). And the increasing of percent parasitemia of P-only and P+VitC mice showed no significantly difference, thus, it is possible that VitC is not able to serve as the anti-malarial drug candidate for erythrocytic stage of the disease. From our finding, there are similarities in colors of livers and spleens in both P-only and P-VitC groups. This might be explained that VitC has no haemozoin clearance property. Study of pathological changes of liver and spleen showed hepatomegaly and splenomegaly in severe malaria infection. (Nacher et al., 2001). Cellular accumulation and congestion within these 2 organs could lead to the heavier weights. From our result of gross examination, P-only mice livers and spleens showed no significant difference in weight if compared with C-mice. This explanation might be

suggested that the liver and spleen of P-only mice are under very severe condition. Part of systemic sequestration and anemia reduce the chance of circulating cells passing into these 2 organs, thus indicating poor prognosis in P-only mice livers and spleens which was in agreement from previous study (Thuma *et al.*, 1998). Interestingly, P-VitC livers and spleens showed similar weights as C mice. It is likely that VitC plays a crucial role during the early phase of malaria infection as for reducing cellular accumulation and congestion and induce circulating cells passing into these 2 organs smoothly. As for size, during malaria infection, liver and spleen are also showed hepatomegaly and splenomegaly in term of increasing in sizes which were the result from inflammation, cytotoxic substances accumulation and white blood cells accumulation. Livers of P-only mice demonstrated no significant difference in size if compared with C mice livers whereas spleen of P-only mice showed significant difference in bigger size than C mice spleen. The explanation of similar size of P-only liver to C mice livers might be from the severity and disruption of most cells. For P+VitC livers, no significant difference in size occurred if compared with C liver. From this phenomenon, it seems that VitC actions on size such reducing inflammation, reducing accumulation of cytotoxic substances and reducing accumulation of active phagocytic cells in liver. However, VitC does not play action in spleen. As displayed in P-only and P-vitC spleens, both of them showed significant difference in sizes if compared with C spleen. The spleens of these 2 groups might have the increase of hyperphagocytotic activity of macrophages and cytotoxic substances accumulation to fight against the infection as similar as the reports that demonstrated previously (Laskin *et al.*, 2010). As for spleen of P+VitC mice showed significantly different in size if compared with C spleen thus it seems that the treatment of VitC into the infected spleen is unlikely functioned. It is likely that VitC does not help reducing the courses of inflammation that occurred in malaria infected- spleen. Taking the results from morphology observation to compare with the results from histology, P+VitC mice liver histology was still showed abnormality. Thus, even VitC seems able to help reducing of weight and size of liver nearly normal appearance, however, VitC could not function on some cellular conditions, for instance, inflammation of cells, cell swelling reduction, in addition, clearing away haemozoins. For spleen histology, interestingly, P+VitC mice spleen showed distinguished white and red pulps with marginal zone which was similar to C mice spleen. It is of interest to explain that VitC seems playing function on splenic

cells and related cells. And megakaryocytes that were noticed in P+VitC mice spleen was the confirmation that this spleen was still function. However, similar as in liver histology, VitC was also unable to clear away haemozoins from spleen. In conclusion, taking the results together, even VitC is unable to completely help on morphologically and histologically changes of P+VitC liver and spleen to nearly normal condition, however, consideration of VitC as the supplement into the future malaria drug candidate is interested.

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