Effect of α -Tocopheryloxy Acetic Acid, a Vitamin E Derivative Mitocan, on the Experimental Infection of Mice with *Plasmodium Yoelii*

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Research

Keywords: α -tocopheryloxy acetic acid, Mice, Plasmodium yoelii, Reactive oxygen species

Posted Date: April 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-457362/v1>

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Abstract

Background: Malaria parasites are known to be vulnerable to oxidative stress. In this study, we examined the effects of α -tocopheryloxy acetic acid (α -TEA), which is a vitamin E analogue mitocan, administration on *Plasmodium yoelii* infection in mice.

Methods: Alpha-TEA was mixed with diet and fed to C57BL/6J mice before and/or after infection. For parasite infection, 4×10^4 *P. yoelii* 17XL-infected red blood cells were inoculated by intraperitoneal injection. In another series of experiment, the effect of the oral administration of α -TEA on *P. yoelii* 17XL infection in mice was examined. Finally, the combined effect of α -TEA and dihydroartemisinin or chloroquine on *P. yoelii* 17XL infection was examined.

Results: When 0.25% α -TEA was mixed with the diet for 7 days before infection and 14 days after infection (in total for 21 days), for 14 days after infection, and for 11 days from the third day after infection, all *P. yoelii* 17XL-infected mice survived during the observation period. However, all control mice died within 12 days after infection. These results indicated that α -TEA functions effectively even when administered post-infection. The oral administration of α -TEA for *P. yoelii* 17XL infection was also significant. Although the infected mice in the solvent control died within 10 days after infection, 90% of the mice infected with *P. yoelii* 17XL survived during the observation period when treated with 10 mg/head/day of α -TEA for 3 days from day 3 after infection. Although the combined effect of α -TEA and dihydroartemisinin (DHA) or chloroquine on *P. yoelii* 17XL infection was significant, no synergistic or additive effects were observed from the survival curve.

Conclusions: This study showed the beneficial effects of α -TEA on the experimental infection of mice with *P. yoelii* 17XL. The stimulatory action of α -TEA on mitochondria and the accompanying reactions, such as reactive oxygen species production, and induction of apoptosis might have some effect on malarial infection.

Background

Malaria is a protozoan infection with fever, anemia, and splenomegaly as the main symptoms. As one of the three major global infectious diseases, about half of the world's population is at risk of malaria. In 2019, there were 229 million cases and 409,000 deaths from malaria. In particular, children under the age of five are most susceptible to malaria, accounting for two-thirds of malaria deaths worldwide [1].

Protozoa, including this malaria parasite, are vulnerable to oxidative stress, and reactive oxygen species (ROS) intermediate the hemolysis of host erythrocytes, endothelial damage, and parasite death in malaria [2, 3]. Vitamin E is a fat-soluble antioxidant and its derivatives produce ROS and exhibits anticancer activity [4, 5]. Alpha-tocopheryl succinate (α -TOS), a derivative of vitamin E, showed anticancer activity against HER2-positive breast cancer in mice [6, 7]. For malaria infection, administration of 50–100 mM of α -TOS on days 1, 3, 5, and 7 after *P. yoelii* 17XL or *Plasmodium berghei* ANKA infection resulted in a significant increase in host survival in mice [8]. However, since this chemical compound is decomposed

by an esterase it has low stability and clinical application may be challenging. Therefore, in this study, we focused on α -tocopheryloxy acetic acid (α -TEA), which is a more stable vitamin E derivative [9], and examined the effects of administration and concomitant use with existing drugs on *P. yoelii* infection in mice. Alpha-TEA is a vitamin E derivative derived and synthesized from α -tocopherol that has an ether bond and is not decomposed by esterase, thus, it can be taken orally [10, 11].

Methods

C57BL/6J mice were purchased from Japan CLEA (CLEA, Tokyo, Japan) and bred in a specific pathogen-free facility. Experimental infection with *P. yoelii* was performed using male mice at a biosafety level 2 facility. The room temperature ($24 \pm 1^\circ\text{C}$) and humidity ($50 \pm 10\%$) were regulated, and lighting was controlled (lights on from 7:00 to 19:00). Mice had free access to water and a commercial regular diet (CA-1; CLEA Japan, Tokyo, Japan). The animals used in this study were treated and cared for based on the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine. All animal experimental protocols were approved by the Institutional Animal Ethics Committee, Obihiro University of Agriculture and Veterinary Medicine (Approval number #20–125).

Alpha-TEA distributed by Eisai (Eisai Co., Ltd., Tokyo, Japan) were mixed with diet (0.083% and 0.25% (w/w) of α -TEA) and fed to C57BL/6J mice for 7 days before infection and 14 days after infection (in total for 21 days), for 14 days after infection, and for 11 days from day 3 after infection. Hahn et al. examined the antitumor effect of dietary administration of α -TEA in mice, and showed that α -TEA had a significant dose-dependent antitumor effect at a dose of 0.05 to 0.3% [12]. Based on this report, 0.083% (low dose) and 0.25% α -TEA diet (high dose) groups were provided in our present study. In order to examine the effect of prophylactic administration of the drug, an experimental group was provided as pre- and post-infection administration. The preparation of the mixed-diet was outsourced to CLEA Japan). For parasite infection, 4×10^4 *P. yoelii* 17XL-infected red blood cells (iRBCs) were inoculated by intraperitoneal injection in α -TEA-treated mice and control mice fed a normal diet, and their parasitemia and survival rates were monitored. The day of infection was defined as day 0. From day 3 after infection, 2 μL of peripheral blood was collected from the tip of the tail, smeared on a glass slide, stained with Giemsa, and the proportion of infected erythrocytes in total erythrocytes (parasitemia) was determined using a phase-contrast microscope (DIAPHOTO-TMD300, Nikon, Tokyo, Japan).

In another series of experiment, the effect of the oral administration of α -TEA on *P. yoelii* 17XL infection in mice was examined. Alpha-TEA was dissolved with 20% DMSO (Dimethyl sulfoxide, Sigma-Aldrich, Tokyo, Japan) in PEG-300 (Polyethylene Glycol 300, Wako, Osaka, Japan). Mice were infected with 4×10^4 *P. yoelii* 17XL iRBCs and orally administered 0, 1, 3, and 10 mg/head/day of α -TEA from days 3 to 5 after infection, and their parasitemia and survival rate were monitored.

Finally, the combined effect of α -TEA and dihydroartemisinin (DHA) or chloroquine on *P. yoelii* 17XL infection was examined. Mice were infected with 4×10^4 *P. yoelii* 17XL iRBCs and orally administered 3

mg/head/day of α -TEA and intraperitoneally injected with 1 mg/kg/day of DHA (D3793, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) or 1 mg/kg/day of chloroquine (C6628, Sigma-Aldrich Japan, Tokyo, Japan) from days 3 to 5 after infection. When the combined effect of the two compounds was examined, the doses at which a survival rate of about 50% was observed when each drug was administered alone were used [13, 14].

As the control, a solvent control group (0 mg α -TEA) in which the parasite and the solvent were administered and a control group in which only the parasite was administered were provided. Each infectious experiment was repeated at least three times and showed typical and representative results. Parasitemia was analyzed using the One-way ANOVA and Tukey test. Survival rate was analyzed using the log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests implemented in GraphPad Prism 5. For all analyses, a value of $p < 0.05$ was considered statistically significant.

Results

As shown in Fig. 1, the effect of α -TEA mixed with diet on *P. yoelii* 17XL infection was significant. When 0.25% α -TEA was mixed with the diet for 7 days before infection and 14 days after infection (in total for 21 days), for 14 days after infection, and for 11 days from the third day after infection, all *P. yoelii* 17XL-infected mice survived during the observation period. However, all control mice died within 12 days after infection. Although the effect of lower concentrations of α -TEA (0.083%) was limited, the survival rate remained significantly higher than that of the control group ($P < 0.05$). Regarding parasitemia (Fig. 2), the control and 0.083% groups showed almost the same changes in proportions of parasitemia after infection, and there was no significant difference between the two groups ($P > 0.05$). When 0.25% α -TEA was administered for 7 days before infection and 14 days after infection (in total for 21 days), proportions of parasitemia were significantly higher than when 0.25% α -TEA was administered for 14 days after infection on days 8, 10, 12, and 14 after infection ($P < 0.05$), and for 11 days after infection on days 4, 8, 10, 12, and 14 after infection ($P < 0.05$). Therefore, it was clarified that α -TEA functions effectively even when administered post-infection.

As shown in Fig. 3, the oral administration of α -TEA for *P. yoelii* 17XL infection was also significant. Although the infected mice in the solvent control (0 mg α -TEA) died within 10 days after infection, 90% of the mice infected with *P. yoelii* 17XL survived during the observation period when treated with 10 mg/head/day of α -TEA for 3 days from day 3 after infection. The survival rates of the 10 mg/head/day group were significantly higher than those in the 1 and 3 mg/head/day groups ($P < 0.05$). The survival rates of the 3 and 10 mg/head/day groups were significantly higher than that of the solvent control group ($P < 0.05$). In our preliminary experiment, when the infected mice were treated with α -TEA for 3 days from day 5 after infection, the survival rate decreased to about half that of the treatment from the third day (data not shown). For parasitemia (Fig. 4), although all experimental groups showed an increase in the proportion of parasitemia after infection, the proportions in the 3 and 10 mg/head/day groups on day 6 after infection were significantly lower than in the solvent control group ($P < 0.05$). In addition, the

proportion of parasitemia in the 10 mg/head/day group was significantly lower than those in the 1 and 3 mg/head/day groups on days 6, 8, and 19 after infection ($P < 0.05$).

Figure 5 shows the combined effect of α -TEA and DHA on survival after *P. yoelii* 17XL infection in mice. There was no significant difference in survival rate after infection between the solvent and untreated controls. All solvent control mice and non-treated control mice died by day 9 and 23 post-infection, respectively. When mice were administered both α -TEA (3 mg/head/day) and DHA (1 mg/kg/day) for 3 days from day 3 after infection, they showed higher survival compared with both the solvent and non-treated control groups ($P < 0.05$), but not the α -TEA ($P = 0.06$) and DHA ($P > 0.05$) groups. As shown in Fig. 6, parasitemia remained similar in all experimental groups, but there was a significant difference between DHA and the combination group on day 4 ($P < 0.05$).

As shown in Fig. 7, although the combined effect of α -TEA and chloroquine on *P. yoelii* 17XL infection was significant, no synergistic or additive effects were observed from the survival curve. Namely, survival after the combined administration of α -TEA (3 mg/head/day) and chloroquine (1 mg/kg/day) for 3 days from day 3 after infection was significantly higher than that in both controls and chloroquine ($P < 0.05$), but not in the single administration of α -TEA ($P > 0.05$). In the transition of parasitemia (Fig. 8), although the combination of α -TEA and chloroquine tended to be similar to the α -TEA and chloroquine alone groups. The proportions of parasitemia in the α -TEA and chloroquine combination groups were significantly lower than that in the control group on day 6 after infection ($P < 0.05$).

Discussion

This study showed the beneficial effects of α -TEA on the experimental infection of mice with *P. yoelii* 17XL. There have been some studies regarding the anti-tumor effects of α -TEA [15–21], but the anti-pathogen or anti-protozoal effects have not yet been investigated. When *P. yoelii* 17XL-infected mice were treated with α -TEA via being mixed into the diet or through oral administration, their viability was almost completely maintained (Figs. 1 and 3) and the parasites were eliminated from their blood (Figs. 2 and 4). However, the effect of pretreatment with α -TEA on parasitemia was limited. Specifically, there was no difference in survival rates whether administration commenced before or after infection, although there was a difference in the transition of parasitemia. When comparing the groups that received α -TEA either before or after infection, the latter significantly reduced parasitemia on days 8, 10, 12, 14, and 19 after infection (Fig. 2, $P < 0.05$). These data indicate that it may be possible to maintain parasitemia lower by increasing the blood α -TEA concentration just before the elevation of parasitemia after malarial infection. Furthermore, α -TEA may act directly on the protozoa rather than modifying the host environment. It has been reported that the administration of 50–100 mM of α -TOS after *P. yoelii* 17XL infection significantly increases host survival in mice [8]. However, all α -TOS-treated mice died within 20 days of infection, while α -TEA administration rescued almost all infected mice in this study (Figs. 1 and 3). As α -TEA is not hydrolyzed and has good stability [10, 11], it may be an effective anti-malaria drug candidate. In our preliminary pharmacokinetic experiment, in which 0.25% α -TEA-mixed diet was fed to uninfected C57BL/6J mice for 21 days, the average food intake of the mice was 3.6 g/head/day. The daily food

intake was comparable to mice fed with a normal chew diet. From this value the average α -TEA intake of mice is 9 mg/head/day. The elimination half-life ($T_{1/2}$) was 61.3 h after a single oral dose of α -TEA (10 mg/head) in mice; the maximum concentration (C_{max}) was 25.7 μ g/mL and the area under the blood concentration time curve (AUC) was 1,780 μ g·h/mL. In addition, the mice orally administered with α -TEA (10 mg/day) for 3 days had a plasma α -TEA concentration of 10 μ g/mL or higher for 6 days from the start of administration (Kasai et al., unpublished data).

When DHA was combined with α -TEA for *P. yoelii* 17XL infection, no synergistic effect was observed. However, α -TEA and DHA did not antagonize, and there may have been an additive effect (Fig. 5). The survival rate on day 30 was 0% in the 3 mg α -TEA and 40% in the 1 mg DHA group, whereas this was 60% in the DHA (1 mg) with α -TEA (3 mg) group. The survival rate by day 30 in the DHA with α -TEA group was significantly higher than that in the control group ($P < 0.05$). Although there was no difference between the DHA with α -TEA group and DHA ($P > 0.05$) and α -TEA group ($P = 0.06$), the survival rate tended to increase in the combined group. On the other hand, the combined use of α -TEA and chloroquine in *P. yoelii*-infected mice did not have a clear combined effect on their survival rate (Fig. 7). In the combined group, parasitemia increased sharply after the end of the administration period (Fig. 8), suggesting that the combination effect may be exerted more strongly by extending the administration period.

It remains unclear whether anti-malarial effects of α -TEA were caused by impact on parasites directly, regulation of pathological mechanisms or enhancement of host defense mechanisms such as the immune system. However, parasites might certainly intake α -TEA from plasma and erythrocyte membrane. In our preliminary experiment, the number of trypanosome was significantly lower following α -TEA treatment than that after control treatment (no α -TEA) in culture *in vitro*, suggesting that α -TEA directly affected trypanosome. α -TEA induced the production of ROS, such as hydroxyl radical and peroxynitrite, in protozoans. (Kawamura et al., unpublished observation). Oxidative stress may inhibit the development of parasite growth. Alpha-TEA stimulates mitochondria to produce ROS and induces the apoptosis of tumor cells [16, 20, 22] as well as enhances the anti-tumor activity of trastuzumab against HER2/neu-expressing breast cancer [17, 18]. As α -TEA had a stronger inhibitory effect on breast cancer than α -TOS, and is more stable in plasma [9], it is expected that the stimulatory action of α -TEA on mitochondria [23] and the accompanying reactions, such as ROS production, induction of apoptosis, and stimulation of autophagy [19] might have some effect on malarial infection. Parasites exposed to α -TEA would readily accumulate ROS by interfering with the mitochondrial redox chain and activating the intrinsic apoptotic pathway. Endothelial cells deficient in mitochondrial DNA are resistant to α -TEA, both in ROS accumulation and apoptosis induction, maintaining their angiogenic potential [24]. Furthermore, α -TEA is a potent anti-tumor agent with a safe toxicity profile in mice [25] and dogs [26].

Conclusion

In this study, α -TEA was effective against murine malaria, but no combination effect with DHA or chloroquine was observed. Since a sufficient antimalarial effect can be obtained with α -TEA alone, future studies could focus on the combined effect of α -TEA with other existing drugs and consider a drug with a

more combined effect. The prophylactic antimalarial activity of premedication with α -TEA may also be interesting. Furthermore, it is expected that this compound will be developed as an antiprotozoal drug by analyzing the mechanism of action of α -TEA and examining its effect on other protozoan infections in future.

Declarations

Consent for publication: Not applicable.

Funding: A part of this work was supported by grant from the Naito Foundation.

Conflicts of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval: The animals used in this study were treated and cared for based on the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine. All animal experimental protocols were approved by the Institutional Animal Ethics Committee, Obihiro University of Agriculture and Veterinary Medicine.

Availability of data and material: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions: The research was designed by H.S., A.K. and S.K. Laboratory experiments were performed by K.K. and A.K. The manuscript was written by H.S. and R.U-S.

Acknowledgments: We would like to thank Editage (www.editage.com) for English language editing.

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Figures

Fig 1

Figure 1

Effect of α -TEA mixed with diet on survival after *P. yoelii* 17XL infection in mice. In total, 0.083% and 0.25% (w/w) α -TEA were mixed with diet and fed to C57BL/6J mice for seven days before 4×10^4 iRBCs and 14 days after infection (0.083%-21, 0.25%-21), for 14 days after infection (0.25%-14), and for 11 days from the third day after infection (0.25%-11). Control vs. 0.083%-21; $P < 0.05$. Control vs. 0.25%-21, -14, and -11; $P < 0.05$. 0.083%-21 vs. 0.25%-21, -14, and -11; $P = 0.05$

Fig 2

Figure 2

Effect of α -TEA mixed with diet on parasitemia after *P. yoelii* 17XL infection in mice. In total, 0.083% and 0.25% (w/w) α -TEA were mixed with diet and fed to C57BL/6J mice for 7 days before 4×10^4 iRBCs and 14 days after the infection (0.083%-21, 0.25%-21), for 14 days after infection (0.25%-14), and for 11 days from day 3 after infection (0.25%-11). Error bars indicate standard error. Control vs. 0.25%-21, -14, and -11 on day 8; $P < 0.05$. 0.083%-21 vs. 0.25%-21 on days 4, 6, 8, 12, 14, and 19; $P < 0.05$. 0.083%-21 vs. 0.25%-14 on days 6 and 8, 10, 12, 14, and 19; $P < 0.05$. 0.083%-21 vs. 0.25%-14 on days 8, 10, 12, and 14; $P < 0.05$. 0.25%-21 vs. 0.25%-11 on days 4, 8, 10, 12, and 14; $P < 0.05$. 0.25%-14 vs. 0.25%-11 on day 4; $P < 0.05$

Fig 3

Figure 3

Effect of oral administration of α -TEA on survival after *P. yoelii* 17XL infection in mice. C57BL/6J mice infected with 4×10^4 iRBCs were orally administered 0, 1, 3, and 10 mg/head/day of α -TEA for 3 days from day 3 after infection. As the control, a solvent control group (0 mg α -TEA) in which the parasite and the solvent were administered was provided. 10 mg/head/day vs. 0, 1, and 3 mg/head/day; $P < 0.05$. 3 mg/head/day vs. 0 mg/head/day; $P < 0.05$

Fig 4

Figure 4

Effect of the oral administration of α -TEA on parasitemia after *P. yoelii* 17XL infection in mice. C57BL/6J mice infected with 4×10^4 iRBCs were orally administered 0, 1, 3, and 10 mg/head/day of α -TEA for 3 days from day 3 after infection. As the control, a solvent control group (0 mg α -TEA) in which the parasite and the solvent were administered was provided. Error bars indicate standard error. 0 mg/head/day vs. 3 mg/head/day on day 6; $P < 0.05$. 0 mg/head/day vs. 10 mg/head/day on days 6 and 8; $P < 0.05$. 1 mg/head/day vs. 10 mg/head/day on days 6, 8, and 19; $P < 0.05$. 3 mg/head/day vs. 10 mg/head/day on days 6, 8, 14, and 19; $P < 0.05$

Fig 5

Figure 5

The combined effect of α -TEA and DHA on survival after *P. yoelii* 17XL infection in mice. C57BL/6J mice infected with 4×10^4 iRBCs were administered 3 mg/head/day of α -TEA and 1 mg/kg/day DHA for 3 days from day 3 after infection. As the control, a solvent control group (0 mg α -TEA) in which the parasite and the solvent were administered and a control group in which only the parasite was administered were provided. Control and solvent vs. α -TEA + DHA; $P < 0.05$. Solvent vs. DHA and α -TEA; $P < 0.05$. α -TEA vs. α -TEA + DHA; $P = 0.06$

Fig 6

Figure 6

The combined effect of α-TEA and DHA on parasitemia after *P. yoelii* 17XL infection in mice. C57BL/6J mice infected with 4×10^4 iRBCs were administered 3 mg/head/day of α-TEA and 1 mg/kg/day DHA for 3 days from day 3 after infection. As the control, a solvent control group (0 mg α-TEA) in which the parasite and the solvent were administered and a control group in which only the parasite was administered were provided. Error bars indicate standard error. DHA vs. α-TEA + DHA on day 4; $P < 0.05$

Fig 7

Figure 7

The combined effect of α -TEA and chloroquine on survival after *P. yoelii* 17XL infection in mice. C57BL/6J mice infected with 4×10^4 iRBCs were administered 3 mg/head/day of α -TEA and 1 mg/kg/day chloroquine for 3 days from day 3 after infection. As the control, a solvent control group (0 mg α -TEA) in which the parasite and the solvent were administered and a control group in which only the parasite was administered were provided. Control, solvent, and chloroquine vs. α -TEA + chloroquine; $P < 0.05$. Control vs. α -TEA; $P = 0.05$. Solvent vs. α -TEA; $P < 0.05$. α -TEA vs. chloroquine; $P < 0.05$

Fig 8

Figure 8

The combined effect of α -TEA and chloroquine on parasitemia after *P. yoelii* 17XL infection in mice. C57BL/6J mice infected with 4×10^4 iRBCs were administered 3 mg/head/day of α -TEA and 1 mg/kg/day chloroquine for 3 days from day 3 after infection. As the control, a solvent control group (0 mg α -TEA) in which the parasite and the solvent were administered and a control group in which only the parasite was administered were provided. Error bars indicate standard error. Control vs. α -TEA + chloroquine on days 6, 10, and 19; $P < 0.05$. Control vs. α -TEA on day 8; $P < 0.05$