Article

Evaluation of 5-Aminolevulinic Acid Plus Ferrous Ion for Its Potential to Improve Symptoms of Bovine Babesiosis

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Abstract: 5-aminolevulinic acid (ALA), an endogenous, non-proteinogenic, naturally occurring amino acid found in diverse organisms, is a precursor of heme biosynthesis. For apicomplexan protozoan parasites, an ALA and sodium ferrous citrate (SFC) combination was previously evaluated and suggested as a potential drug candidate for *Plasmodium falciparum* malaria. This study aimed to evaluate the potential of this combination against bovine babesiosis. ALA administration at 100 and 500 μ M coupled with 10 μ M SFC in culture medium significantly inhibited intraerythrocytic development and growth of *Babesia bovis*, which causes cerebral babesiosis in cattle, under in vitro culture. However, administration of 10 μ M SFC only in the medium did not inhibit parasite growth. ALA/SFC was efficacious in treating babesiosis in an experimental animal model with *B. microti*, which causes debilitating babesiosis in mice. Female BALB/c mice were infected with *B. microti* and administered a single oral dose of ALA/SFC combination daily in different concentrations for 30 days. Treatment with ALA/SFC at 4/0.4 mg/kg body weight significantly suppressed parasite development in the mice blood circulation and resulted in significantly lower parasitemia. Moreover, body weight loss in the mice has been improved significantly compared with the control group at the peak of parasitemia. Treated mice showed moderate decreases in red blood cell count, hemoglobin value, and hematocrit compared with those observed in the control group, indicating an effect in moderating progressive anemia. These findings suggested the potential of ALA/SFC to achieve symptomatic improvement against bovine babesiosis.

Keywords: Apicomplexa; babesia microti; bovine babesiosis; 5-ALA

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1. Introduction

Babesiosis is a tick-borne disease caused by intraerythrocytic protozoan parasites of the genus *Babesia*. In particular, *Babesia bovis* infection causes a severe disease characterized by hemolytic anemia and high mortality in adult bovines [1], whereas *B. microti* invades and replicates in red blood cells (RBCs) of animals, including humans, to cause zoonotic babesiosis [2]. Infection of *B. microti* in mouse has been used as an experimental infection model to evaluate the effect of potential compounds for the treatment of bovine babesiosis [3,4]. The control strategies currently applied for bovine babesiosis include treatment of the infected animal, tick control using acaricide and live attenuated vaccine administration, in which emerging resistance to drugs and acaricides and lack of effective vaccines are the main obstacles [1,5]. *Babesia* spp., along with many other species (e.g., *Plasmodium* spp. and *Toxoplasma* sp.), belong to the phylum Apicomplexa. Most apicomplexan parasites have a non-photosynthetic plastid called the apicoplast, which is involved in some

essential metabolic pathways such as the heme biosynthesis pathway, and its functions are considered to be potential targets for chemotherapy [6,7].

5-Aminolevulinic acid (ALA) is an endogenous, non-proteinogenic, naturally occurring amino acid found in diverse organisms such as bacteria, fungi, plants, and animals, and is a precursor of heme biosynthesis. It is commercially available as a component of cosmetics, dietary supplements, and pharmaceuticals for cancer diagnosis and therapy [8,9].

Amongst protozoan parasites, ALA has only been evaluated against *Plasmodium falciparum* infection. ALA was recently reported to efficiently inhibit in vitro growth of the human malaria parasite *P. falciparum* in the presence of ferrous ion (Fe^{2+}) [10]. Reactive oxygen species (ROS) are produced by interaction between the ALA-derived heme intermediates and Fe^{2+} , and ROS can damage DNA and membranes of the apicoplast. Therefore, ALA was suggested to be a potential antimalarial treatment in humans [10]. In addition, the combination of ALA and sodium ferrous citrate (SFC) as a source of ferrous ions was found to be beneficial in improving symptoms in malaria patients, which led to fast recovery, thus suggesting ALA/SFC as a promising composition for malaria chemotherapy [9].

Accordingly, this study was conducted to evaluate the growth inhibitory effect of the ALA/SFC combination against *B. bovis* under in vitro culture conditions. In addition, the curative effect of orally administered ALA/SFC in mice against *B. microti* infection was investigated to evaluate the potential of ALA/SFC to offer symptomatic improvement of bovine babesiosis.

2. Results

2.1. In Vitro Study

The addition of 100 and 500 μM ALA with 10 μM SFC in the culture medium significantly inhibited *B. bovis* growth. However, the addition of 10 μM SFC alone in the medium did not inhibit parasite growth (Figure 1). The addition of 1 μM diminazene aceturate (DA), which was anti-babesial drug [3], in the medium completely inhibited parasite growth (Figure 1).

Figure 1. In vitro growth inhibition of *Babesia bovis* by ALA/SFC. *B. bovis* cultures were incubated with SFC alone, different concentrations of ALA/SFC combination, or DA in triplicate using 24-well culture plates for 3 days, and the number of parasitized RBCs per 1000 RBCs was counted as the “parasite count” for each well. The representative results of 2 independent experiments are shown. The parasite count for the culture with non-treatment (A), 10 μM SFC alone (B), 100 μM ALA plus 10 μM SFC (C), 500 μM ALA plus 10 μM SFC (D) and 1 μM DA (E) are shown as the mean + S.D. of 3 independent counts of 3 wells. * The differences between (A) and (C)/(D) were significant ($p < 0.05$).

2.2. In Vivo Study

All of the mice infected with *B. microti* survived. The parasite was detected in the blood circulation of all mice for the first time at 2 days post infection (dpi). The peak level of parasitemia (peak) was recorded at 8 dpi, and the parasitemia gradually decreased thereafter until the end of the experiment at 30 dpi (Figure 2). All mice receiving drug administration showed significantly lower parasitemia compared with that in the control mice (group A) at the peak (8 dpi). The mice administered ALA/SFC 4/0.4 mg/kg (group D) showed the lowest parasitemia at the peak (Figure 2).

Table 1. Summary of days post infection (dpi) on which significant differences of each parameter were observed.

Group	Dose (mg/kg) of *		Parameters				
	ALA	SFC	PE	BW	RBC	HGB	HCT
A **							
B		10	8 dpi	11, 17, 20, 23, 26, and 29 dpi			
C	100		8 dpi				
D	4	0.4	5 and 8 dpi	8 dpi	8 dpi	8 dpi	8 dpi
E	20	2	8 dpi	8 dpi			
F	100	10	8 dpi				

* 5-Aminolevulinic acid phosphate (ALA) and sodium ferrous citrate (SFC) were dissolved in phosphate-buffered saline (PBS) for oral administration. ** PBS was administered as control. PE: parasitaemia (%) of group D at 5 and 8 dpi was significantly lower than that of group A (control group). PE of group B, C, E, and F at 8 dpi was significantly lower than that of group A. BW: body weight (percentage to pre-treatment) gains of group B at 11, 17, 20, 23, 26, and 29 dpi were significantly greater than that of group A. BW gains of group D and E at 8 dpi were significantly higher than that of group A. RBC: red blood cell count (percentage to pre-treatment) of group E at 8 dpi was significantly higher than that of group A. HGB: hemoglobin value (percentage to pre-treatment) of group E at 8 dpi was significantly higher than that of group A. HCT: hematocrit percentage (percentage to pre-treatment) of group E at 8 dpi was significantly higher than that of group A.

Figure 2. Effects of ALA/SFC treatments to reduce parasitemia in mice infected with *Babesia microti*. Mice were infected with *B. microti* and orally administered SFC alone or different concentrations of ALA plus SFC or PBS for 30 days post infection (dpi). Five mice were used for each experimental group, and they were treated with PBS (control group) (A), 10 mg/kg SFC (B), 100 mg/kg ALA (C), 4 mg/kg ALA plus 0.4 mg/kg SFC (D), 20 mg/kg ALA plus 2 mg/kg SFC (E), or 100 mg/kg ALA plus 10 mg/kg SFC (F). The parasitemia in each mouse was monitored at day 0 and then every 3 days from 2 dpi. The number of parasitized RBCs per 1000 RBCs was counted in triplicate and the parasitemia (%) was calculated. Each plot is the mean of the triplicate counts. The dpi when a significant difference in parasitemia was observed between (A) and (B)/(C)/(D)/(E) are summarized in Table 1.

After the infection, the mice in the control group (group A) and the mice treated with ALA alone (group C) and ALA/SFC 100/10 mg/kg (group F) lost body weight. The lowest body weight of these animals was recorded at 11 dpi, and it gradually increased thereafter until the end of the experiment (30 dpi) (Figure S1 Body weight). The body weights of the mice treated with ALA/SFC 4/0.4 mg/kg (group D) and ALA/SFC 20/2 mg/kg (group E) continued to increase after the infection and recorded greater gains at 8 dpi compared with those in the control group, although the weights showed transient reduction at 11 dpi (Figure S1 Body weight and Table 1). The mice treated with SFC alone (group B) showed greater gain in body weight compared with that in the other groups throughout the experiment (Figure S1 Body weight and Table 1).

RBC, hemoglobin value (HGB), and hematocrit percentage (HCT) in all mice decreased until 8 dpi in association with the increase in parasitemia (Figure S1 RBC, HGB, and HCT). Only the mice administered ALA/SFC 4/0.4 mg/kg (group D) showed moderate decreases in RBC, HGB, and HCT compared with those in the control group (group A) (Figure S1 RBC, HGB, and HCT and Table 1).

3. Discussion

The growth inhibitory effect of the ALA/SFC combination against *P. falciparum* under in vitro culture condition [10] and its curative potential against *P. berghei* infection in mice [9] have recently been reported. The apicoplast, which is involved in the heme biosynthesis pathway of malaria parasites, is essential for parasite development [7], and ALA was suggested to be a potential antimalarial drug that targets the heme biosynthesis pathway in this organelle [10]. Given that *Plasmodium* and *Babesia* are apicomplexan parasites that both have apicoplasts [6], it could be hypothesized that ALA might have potential in the treatment of bovine babesiosis.

The growth inhibitory efficacy of the combination of ALA/SFC against *B. bovis* was evaluated and found to significantly inhibit parasite growth under an in vitro culture condition. The addition of ALA/SFC 100/10 μ M and ALA/SFC 500/10 μ M into the culture medium inhibited parasite growth to 64% and 54%, respectively, of the control culture without the compounds. These concentrations of ALA/SFC were comparable to those showing >50% inhibitory effect against *P. falciparum* under an in vitro culture condition [10]. The mode of action proposed for ALA/SFC to inhibit the growth of *P. falciparum* is that accumulation of intermediates such as coproporphyrin III and coexisting Fe^{2+} that form the heme biosynthesis pathway produce ROS, which damages the DNA and membrane of the apicoplast. Analysis of the *B. bovis* genome predicted that the heme biosynthesis pathway found in *P. falciparum* was absent in this parasite species [11]. However, the parasite genome contains the gene coding for delta-aminolevulinic acid dehydratase, which catalyzes the reaction producing porphobilinogen from ALA as the second step in the heme biosynthesis [11,12]. This step in *P. falciparum* takes place in the apicoplast [12]. *Babesia* parasites possess several heme-dependent enzymes that require heme as a prosthetic group for their catalytic activities; however, no transporter for heme salvage has been identified so far in the parasite genome [11,12]. Re-evaluation of the genome with appropriate prediction algorithms may provide additional information on the babesia heme biosynthesis pathway and may explain the mode of action of ALA/SFC in this parasite species.

The curative effect of ALA/SFC oral administration in mice was evaluated in vivo with a *B. microti* experimental infection model. Oral administration was chosen because the application method of ALA/SFC as a “feed additive” to animals such as dairy cows and beef cattle was expected. The safety of ALA/SFC as a dietary supplement has been reviewed [8]. All administrations including either ALA or SFC alone or their combinations inhibited development of *B. microti* in the mice blood circulation compared with that in the control (Figure 2 and Table 1). The oral administration of ALA/SFC 4/0.4 mg/kg showed a curative effect, and the dose was much lower than that showed curative effect against the mouse malaria infection (ALA/SFC 300/300 mg/kg) [9]. ALA may have other

modes of action against babesia parasites other than that against malaria parasites. The antiviral activity of ALA against feline infectious peritonitis virus with unidentified factors other than oxidative stress has been reported [13]. The mice administered ALA alone showed significantly lower parasitemia than that of the control. The curative effect of ALA alone at a high dose (600 mg/kg) without SFC was also observed in mice infected with malaria, and it was explained by that the mice had ingested sufficient ferrous ion from their diet under the ordinal experimental condition [9]. The curative effect of ALA alone in our experiment was most probably attributable to the same reason as the animals were ordinarily fed with an experimental animal diet that contained sufficient ferrous ion (34.24 mg of ferrous ion/100 g of CLEA Rodent Diet CE-2; Japan CLEA Inc., Tokyo, Japan, https://www.clea-japan.com/en/products/general_diet/item_d0030 (accessed on 15 October 2021)).

The progression of parasitemia was accompanied by body weight loss in all of the infected mice. The body weight of the mice gradually decreased until the peak of parasitemia was reached and then increased thereafter. The body weight losses at the peak of parasitemia in the mice administered ALA/SFC 4/0.4 mg/kg and ALA/SFC 20/2 mg/kg were significantly improved compared with that of the control mice. It is worth mentioning that administration of SFC alone also significantly improved body weight loss in the mice at the peak of parasitemia and thereafter. The effect of ALA on improving milk protein content has been reported in dairy cows that were fed the compound as a “dietary supplement” [14]. The effect of ALA/SFC administration on the improvement of body weight loss of infected animals is favorable to its application in dairy cows and beef cattle, although the reason for such effect with SFC alone is unclear.

RBC, HGB, and HCT decreased as a result of anemia as the parasitemia progressed. The mice administered ALA/SFC 4/0.4 mg/kg showed moderate decrease in these parameters at the peak of parasitemia compared with the control mice. This finding indicated that oral administration of ALA/SFC could moderate the progression of anemia, one of the most important clinical signs of bovine babesiosis [1].

4. Materials and Methods

4.1. Chemicals

ALA and SFC were obtained from neopharma Japan Co., Ltd. (Tokyo, Japan). Hydrochloric acid (HCl) and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DA was purchased from Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan). ALA was dissolved in ultrapure water (Direct-Q 3UV, Merck Millipore, Darmstadt, Germany) to make a 100 mM solution, and SFC was dissolved in 0.004 N HCl (final concentration) to make a 10 mM solution. Both were filtrated with a 0.2-µm filter (Dismic-25CS, Advantec Co., Ltd., Tokyo, Japan) for sterilization and diluted with the culture medium at the specified concentrations. DA was dissolved in DMSO at 10 mM and stored at 4 °C. For animal experimentation, ALA and SFC were dissolved in phosphate-buffered saline (PBS) containing 0.004 N HCl (final concentration) at the specified concentrations for oral administration. All drugs except 10 mM DA were freshly prepared on the day of use.

4.2. Parasite Culture and Growth Inhibition Assay

B. bovis Texas strain was maintained in culture using a microaerophilic stationary-phase culture system composed of bovine RBCs at 10% hematocrit and GIT medium (FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) [15]. Parasite cultures (1 mL) were incubated with different concentrations of ALA (100 and 500 µM) coupled with SFC (10 µM). The cultures with SFC (10 µM) alone and DA (1 µM) served as iron and anti-babesial drug controls, respectively. The non-treatment control contained DMSO at 0.01%. Each experimental group with initial parasitemia of 0.1% was cultured in triplicate using 24-well culture plate for 3 days. Culture medium with drug was changed daily, and

the number of parasitized RBCs per 1000 RBCs was counted in triplicate on thin smears which had been prepared on the third day of incubation.

4.3. Animals

Six-week-old female BALB/c mice (body weight 19–23 g) were used. The animals were maintained in the animal facilities at the National Research Center for Protozoan Diseases (NRCPD).

4.4. Parasite Infection and Animal Experimentations

The Munich strain of *B. microti*, which has been maintained at the NRCPD, was used [4]. The parasite-infected RBCs (1×10^7) were injected intraperitoneally in each mouse included in the experiment. Five mice were used for each experimental group. One day before infection, body weight and blood parameters (RBC, HGB, and HCT) of each mouse were measured with CellTaq α (Nihon Kohden Corporation, Tokyo, Japan). These parameters in each mouse were then measured every 3 days after infection for 30 days until the end of the experiment. The parasitemia of *B. microti* was monitored every 3 days from 2 days after infection until the end of the experiment by counting the number of parasitized RBCs per 1000 RBCs in triplicate on Giemsa-stained thin blood smears. Administration of the compounds was started on the day of infection with a single oral dose (200 μ L) of the different combinations (Table 1). The administration continued daily for 30 days until the end of the experiment.

The animal experimental protocols were performed with permission from and in accordance with the standards for animal experimentation of Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido Japan (Approval number of Animal exp. 20–33).

4.5. Statistical Analysis

In vitro parasite count, parasitemia, body weight, and blood parameters are expressed as the mean + SD, and values were compared between the control (group A) and the other groups using one-way ANOVA followed by Dunnett's multiple comparisons test for the in vitro and in vivo studies. Differences were considered significant when the P value was less than 0.05. All tests were performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA; www.graphpad.com (accessed on 15 October 2021)).

5. Conclusions

Administration of ALA/SFC showed a growth inhibitory effect against *B. bovis* under an in vitro culture condition. The oral administration of ALA/SFC to mice infected with *B. microti* could suppress parasite development in the blood circulation and improved body weight loss and the anemic condition of the animals. These findings could support the idea of its application as a “feed additive” in dairy cows and beef cattle to moderate the adverse effects of bovine babesiosis in the locations where the disease is endemic.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/parasitologia1040023/s1>, Figure S1: Effects of ALA/SFC treatments to moderate body weight loss and anemia in mice infected with *Babesia microti*.

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