Inhibitory effect of naphthoquine phosphate against *Babesia gibsoni in vitro* and *Babesia rodhaini in vivo*

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Research Article

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Abstract

Background: Drug resistance and severe side effects are major challenges in the treatment of babesiosis as they lead to less choices for treatment. Development of new drugs to enrich the treatment strategies and delay the emergence of drug resistance in parasites is still needed. Naphthoquine (NQ) combined with artemisinin treats *Plasmodium* infection by rapid parasite clearance. The current study repurposed NQ as a babesiosis drug treatment by evaluating the effects of naphthoquine phosphate (NQP) as a single dose treatment for babesiosis.

Methods: *In vitro* anti-*Babesia* activity of NQP was tested on *Babesia gibsoni* cultures. The inhibition of parasite growth was verified using a SYBR green I-based fluorescence assay. *In vivo* efficacy of NQP was evaluated using BALB/c mice infected with *Babesia rodhaini*. The parasitemia level and hematocrit values were monitored.

Results: The half maximal inhibitory concentration of NQP against *B. gibsoni in vitro* was 3.3 ± 0.5 μM. Oral administration of NQP for 5 successive days at a dose of 40 mg/kg of body weight resulted in significant inhibition on parasite growth compared with the control group. All mice in NQP-treated group survived, whereas the mice in control group died between days 6 and 9 post infection.

Conclusion: This is the first study to evaluate the anti-*Babesia* activity of NQP *in vitro* and *in vivo*. The results showed that NQP is a promising drug for babesiosis treatment and drug repurposing may provide new treatment strategies for babesiosis.

Background

Babesiosis is an infectious disease caused by intraerythrocytic parasites of the genus *Babesia.* More than 100 *Babesia* species have been identified. The most notable species include *B. bigemina*, *B. divergens*, and *B. bovis* for bovine, *B. caballi* for equine, *B. canis* and *B. gibsoni* for canine, and *B. microti* and *B. rodhaini* for murine [1]. Moreover, several *Babesia* spp. have been reported to infect humans. Because of its wide range of hosts, babesiosis is one of the most ubiquitous infections of free-living animals and is gaining increasing interest as an emerging zoonosis in humans [1]. The parasites replicate in the mammalian host red blood cells which produce clinical symptoms including fever, hemolytic anemia, anorexia, hemoglobinuria, and emaciation and the severe infection phase often results in death [2]. Current treatment strategies for babesiosis are limited. For instance, imidocarb dipropionate and diminazene aceturate are used for babesiosis in animals, whereas atovaquone combined with azithromycin and clindamycin combined with quinine are used for humans [3]. However, the indiscriminate use and lack of alternative drugs contributed to the emergence of drug resistance in parasites, and reports of adverse side effects are well documented [4, 5].

In the past five decades, the cases of human babesiosis have increased in the United States [6]. A review shows that during 1982 to 1993, 139 hospitalizations occurred due to *Babesia* infection. Among the patients, 25% required intensive care stays and 9 patients died [7]. Moreover, a recent babesiosis

surveillance in the U.S. showed that a total of 7,612 cases of babesiosis were reported to the CDC from 2011 to 2015. Among the cases, 82.5% were classified by the reporting health jurisdiction as confirmed and 17.5% as probable. A total of 7 deaths were attributed to babesiosis and 4 deaths were not babesiosis-related, whereas whether the 35 patients' death were caused by babesiosis were not verified [8]. Atovaquone combined with azithromycin as the standard therapy for babesiosis have reported failures in some clinical cases due to mutations in the *cytochrome b* gene [5]. The atovaquone-azithromycin drug combination is also effective for canine *B. gibsoni* infections. However, a recent study documented the increased detection of a *cytochrome b* gene variant associated with atovaquone resistance in *B. gibsoni* population in dogs in Japan [9].

Although several promising drugs have been reported for babesiosis, such as tafenoquine (TAF) [10, 11], clofazimine [12], and pyronaridine tetraphosphate [13], but due to geographical restrictions, the development of new drugs to enrich the treatment strategies and delay the emergence of drug resistance is still needed.

Naphthoquine (NQ) is an antimalarial drug that was first synthesized in China in 1986 and registered as naphthoquine phosphate (NQP) in 1993. Initial clinical trials showed NQ monotherapy to be highly efficacious without documented toxicity [14, 15]. Subsequently, a drug combination comprised of NQ and artemisinin (ART) at a fixed ratio of 1:2.5 was developed in order to retain the strongpoint of the two drugs and delay the possible emergence of drug resistance. Safety data of NQ-containing therapies involving more than 4000 patients recorded no serious adverse experience, hematology, and biochemistry changes. It has been considered as a promising anti-malarial drug candidate and is marketed under the name of ARCO® in various tropical countries [14-16]. Due to the *Plasmodium* and *Babesia* genera being closely related, we were interested if NQP has effects against the *Babesia* parasite. This study aimed to investigate the inhibitory effects of NQP against *in vitro* cultured *Babesia gibsoni,* a causative agent of canine babesiosis and *in vivo* propagated *Babesia rodhaini*, a highly pathogenic rodent *Babesia* species.

Methods

Chemical reagents

Naphthoquine phosphate (NQP) was purchased from ChemScene (NJ, USA). Tafenoquine (TAF) were purchased from Sigma-Aldrich (Tokyo, Japan). SYBR Green I (SG1) nucleic acid stain was purchased from Lonza America (GA, USA). NQP and TAF were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution with the concentration of 40 mg/ml and diluted with Milli-Q water before use.

Maintenance of the parasites and mouse infection

In this study, we chose *in vitro* cultured *B. gibsoni* as a model to evaluate drug effects on the *Babesia* parasite as it was established and utilized previously in our laboratory [17]. *Babesia gibsoni* Oita strain [18] was cultured and used for the *in vitro* study. Briefly, the parasite was grown using canine red

blood cells (RBCs) suspended in RPMI-1640 supplemented with 20% canine serum and cultured in 24-well plates. For the *in vivo* evaluation, *B. rodhaini* Australia strain [19] was recovered from the stock in our laboratory. For the maintenance of *B. rodhaini*, cryopreserved parasitized-RBCs were passaged by intraperitoneal (i.p.) injection of mice. Challenge infection was performed with i.p. inoculation of 10^7 fresh *B. rodhaini* infected RBCs (iRBCs). A total of 20 BALB/c mice (6-weeks old) were purchased from CLEA Japan and were used to maintain *B. rodhaini* for the *in vivo* study.

In vitro B. gibsoni growth inhibition assay

To test the activity of NQP against *B. gibsoni*, SYBR Green -based fluorescence assay was performed as previously reported [20]. Briefly, *in vitro* cultures of *B. gibsoni* were diluted to 1% parasitemia with fresh canine RBCs. The NQP was dissolved with medium at concentrations that ranged from 0.1 to 5 μM, and incubated with infected RBCs (iRBC) in triplicate in 96-well plates with 5% hematocrit for 96 hours. The fluorescence values were evaluated using the fluorescence spectrophotometer (485 and 518 nm, Fluoroskan Ascent, USA) and the inhibition activity and half inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism 8 (GraphPad Software Inc., USA).

In vivo inhibitory effect of NQP on *B. rodhaini*

Fifteen BALB/c mice intraperitoneally challenged with 10^7 *B. rodhaini* (iRBCs) were randomly assigned to groups (n = 5 per group). When parasitemia was about 3% to 5%, the drug treatment was initiated. The first group received 40 mg/kg of NQP orally for 5 consecutive days while the second group received 20 mg/kg of TAF orally for one time [10-12, 21]. The control group received 5% DMSO in Milli-Q water. Parasitemia was calculated by counting infected RBCs among 3000 RBCs using Giemsa-stained blood smears. Hematocrit (HCT) changes was monitored as an index for the development of anemia by using hematology analyzer (Nihon MEK-6450, Nihon Kohden Corporation, Tokyo, Japan) every 2 days. On day 42 post infection, all survived mice were euthanized. All animal experiments were approved by the Animal Welfare Committee (approval no. 20-128) and were conducted in accordance with the standards for the care and management of experimental animals as stipulated by the Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

Statistical analysis

Data analysis was performed using GraphPad Prism. Differences in parasitemia between the control and treated groups were determined by one-way ANOVA analysis plus Tukey-Kramer *post hoc* analysis. Survival rates were calculated using the Kaplan–Meier method, with regard to the log-rank test. A P value < 0.05 was considered statistically significant.

Results

NQP inhibits *B. gibsoni* growth *in vitro*

The inhibitory effect of NQP was carried out using *in vitro* cultured *B. gibsoni.* NQP significantly inhibited ($P < 0.05$) parasite growth at 2.5 μM and showed inhibition in a dose-dependent manner. The IC_{50} value of NQP on *B. gibsoni* was 3.3 ± 0.5 μM. Meanwhile, the IC_{50} value of the control drug TAF was 20.0 ± 2.4 μM (Fig. S1). The results indicate that NQP has potential efficacy against *Babesia* parasite growth.

In vivo effects of NQP on *Babesia* infection in mouse model

The *B. rodhaini*-infected model was used to further evaluate the effects of NQP on *Babesia* parasite *in vivo*. The highest parasitemia of *B. rodhaini* infected mice in DMSO treated group was 78.2% and all mice died within 10 days post infection (dpi). In contrast, the parasitemia in NQP- and TAF-treated group were significantly ($P < 0.05$) decreased after treatment and showed 95.1% (3.8% peak parasitemia) and 95.8% (3.3% peak parasitemia) inhibition compared to the DMSO-treated group, respectively. Parasitemia was undetectable via Giemsa-staining method in mice treated with NQP and TAF at 10 dpi and 8 dpi, respectively. However, regrowth of parasites was observed in both NQP- (n = 2/5) and TAF- (n = 1/5) treated group on 16 dpi and 22 dpi (Fig. 1a). HCT change as an index for anemia development was monitored. Significant ($P < 0.05$) reductions were observed in DMSO-treated group from 6 dpi compared with the values recorded from the NQP- or TAF-treated group (Fig. 1b). All the mice in NQP- and TAF-treated group survived by 40 dpi ($P = 0.0002$, Fig. 1c). The Giemsa-stained blood smears of DMSO-treated group from 4 dpi to 7 dpi (8 and 9 dpi are not available), NQP-treated group and TAF-treated group from 4 dpi to 8 dpi are shown in Fig. 2. Compared to the DMSO-treated group, TAF-treated group showed an aberrant parasite phenotype and only faint chromatin staining was visible within 2 days of TAF treatment. Unlike the TAF-treated group, NQP treatment showed a significant degeneration of parasites at 8 dpi.

Discussion

Because of the increasing number of treatment failures and toxic effects by using current therapies for babesiosis, exploration and discovery of novel drugs with anti-*Babesia* activity is needed [3, 4, 22]. *Babesia* and *Plasmodium* share similar features in their biology as they are closely related genera among the apicomplexan parasites [23]. Due to this, some anti-*Babesia* drugs were initially investigated because of their antimalarial activity. Several showed excellent activities against *Babesia*, such as atovaquone combined with azithromycin for human babesiosis, and TAF, which was recently investigated for human and canine babesiosis [10, 24, 25].

NQ is a 4-aminoquinoline antimalarial drug with a longer half-life but slower action than ART, and is currently combined with ART to treat malaria [14]. This combination therapy also inhibits *Schistosoma mansoni* [26]. Moreover, NQP combined with azithromycin for malaria treatment was also developed

[27]. The toxic effects of NQP on mammalian host have been reported. Daily treatment in dogs for 14 days at a dose of 17.5 mg/kg/day and in rats for 70 mg/kg/day were considered safe. The safe doses in canine and rat models are equivalent to approximately 10 mg/kg/day in humans [15, 28]. A previous study reported that the concentration of NQP reached the peak level in plasma 2 hours post treatment with a dose of 10 mg/kg. The peak concentrations were 300.84 ng/mL and 273.29 ng/mL in plasma and erythrocytes, respectively, and the half-life of NQP was 198 hours (~8 days) in normal mice [29]. Interestingly, the concentrations were far greater in *P. berghei*-infected mice [29]. The anti-parasitic activity of NQP as demonstrated by the inhibition of *B. gibsoni in vitro* (Fig. S1) prompted us to further explore its anti-*Babesia* activity *in vivo*. We used the lethal species *B. rodhaini* in the mouse model for evaluating NQP as a therapeutic. In the current *in vivo* trial, NQP exhibited excellent inhibitory efficacy as evidenced by reduced parasite growth (Fig. 1a) and degenerative morphological changes in the parasites (Fig. 2). Furthermore, the first 2 days of treatment with 40 mg/kg NQP prevented the rise of *B. rodhaini* parasitemia starting from day 6 post infection compared to the typical rise of mean parasitemia in DMSO-treated mice. In addition, the accumulation of NQP in plasma [29] by completion of the 4-day treatment resulted in morphological changes of parasites in all treated mice at day 8 post infection. Moreover, the TAF-treated group showed an aberrant parasite phenotype (Fig. 2) which has been associated with oxidative stress [10]. *B. rodhaini*-infected mice in DMSO-treated group developed rapid anemia, whereas NQP and TAF prevented anemia development in infected mice (Fig. 1b).

Since TAF was approved by the U.S. Food and Drug Administration (FDA) as a single drug treatment for malaria, TAF studies have attracted much attention [10, 11]. Nonetheless, the limitation of TAF is the risk of inducing severe hemolytic anemia in individuals with G6PD deficiency in humans and the relapse of parasites, which are well documented [10, 24, 30]. Moreover, a single treatment of TAF on immunocompromised hosts could not eliminate parasites [10, 24]. Recently, TAF showed strong and broad anti-parasitic activity against *Babesia* spp., including *B. microti*, *B. gibsoni*, and *B. rodhaini* [10]. Hence, TAF was selected as a reference drug in this study. In the present study, the relapse of parasites was observed both in NQP-treated group and TAF-treated group (Fig. 1a). Therefore, NQP may need to be accompanied by other anti-*Babesia* drugs to augment its effect and prevent the regrowth of parasites.

In addition, the mechanism of action of NQP has not been fully elucidated. The inhibition activity of NQP for *Plasmodium* was hypothesized to be through the inhibition of hemozoin biocrystallization in the digestive vacuole of late-stage parasites and disruption of membrane system. Due to *Babesia* not producing hemozoin during parasite development, the inhibitory effect of NQP on the *Babesia* parasite is hypothesized to be related to targeting the parasite's membrane system [15, 31].

It should be noted that there are some limitations to the present study. Although NQP exhibited a potential antibabesial effect, it has a slower onset of action and a longer half-life, which may easily lead to drug build-up with increasing the probability of developing resistance. Therefore, future studies are warranted to analyze the possible synergistic effect of NQP when administrated in combination with other drug which has a rapid onset of babesicidal action and a short half-life. Such analysis will help to determine

the most effective composition ratio for treatment of *Babesia* in animals in clinical applications. Furthermore, the mode of action by which NQP inhibits the *in vitro* and *in vivo* growth of *Babesia* is still unknown. Subsequently, further studies are required to elucidate this point. Although the present study demonstrated the potential antibabesial efficacy of NQP in a mouse model, additional *in vivo* experiments are required to confirm such inhibitory effect in *B. gibsoni*- infected dog.

Conclusion

The present study demonstrated the inhibitory effect of NQP against *B. gibsoni in vitro* and *B. rodhaini in vivo.* Our findings indicate that NQP is a potential candidate for babesiosis treatment and suggest further investigation on the possible use of this chemical for human babesiosis.

Abbreviations

NQ: naphthoquine; NQP: naphthoquine phosphate; ART: artemisinin; TAF: tafenoquine; SG1: SYBR Green I; DMSO: dissolved in dimethyl sulfoxide; RBCs: red blood cells; iRBCs: infected red blood cells; i.p.: intraperitoneal; IC_{50}: half inhibitory concentration; SD: standard deviation; dpi: days post infection; HCT: hematocrit.

Declarations

Ethics approval and consent to participate

All procedures were carried out according to the ethical guidelines approved by the Obihiro University of Agriculture and Veterinary Medicine (permit numbers: animal experiment, 20-128; DNA experiment, 1723-4 and 1724-4; pathogen, 201712-5).

Consent for publication

Not applicable

Availability of data and material

All data sets were presented as tables, figures and text description in this article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SJ and XX designed the study. SJ carried out the experiments. JL, IZ and YH contributed reagents/materials preparation. SJ, ML, MAR, BT and EMG wrote the manuscript and all authors read and approved the final manuscript.

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Not applicable

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Figures

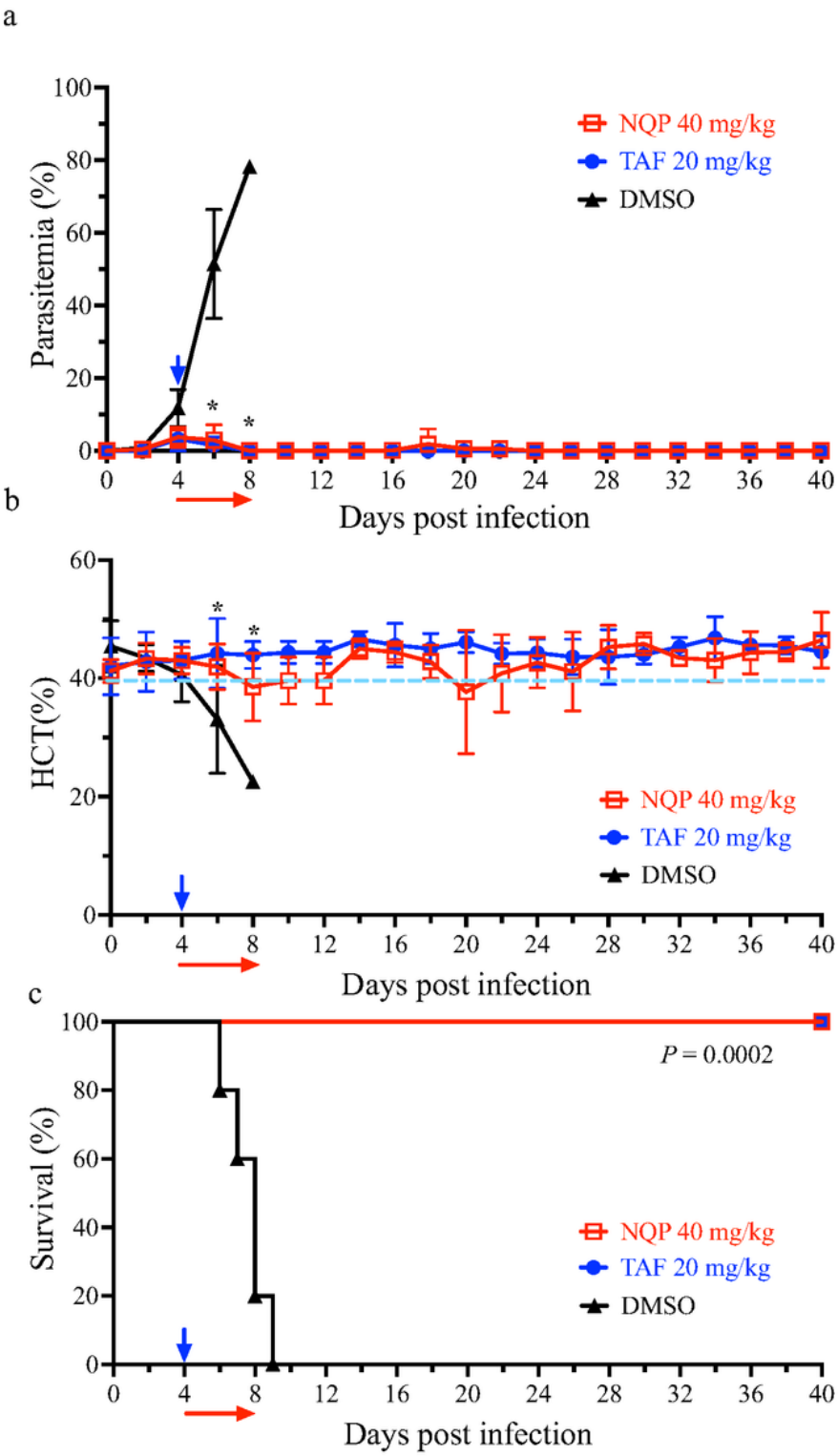

Figure 1

The inhibitory effects of NQP on B. rodhaini in BALB/c mice. (a) The growth inhibition of NQP and TAF as compared with the untreated group. (b) Changes of hematocrit values in mice treated with NQP or TAF as compared with the DMSO-treated group. The asterisks indicate a significant difference ($P < 0.05$) between the NQP- or TAF-treated group and the DMSO-treated group. The arrows indicate time of treatment. (c) Survival rates of NQP and TAF after treatment. The asterisks indicate a significant difference ($P < 0.05$)

between the NQP- or TAF-treated group and DMSO-treated group. The arrows indicate time of treatment. Parasitemia was calculated by counting infected RBCs among 3000 RBCs using Giemsa-stained blood smears. Dotted line indicates reference range.

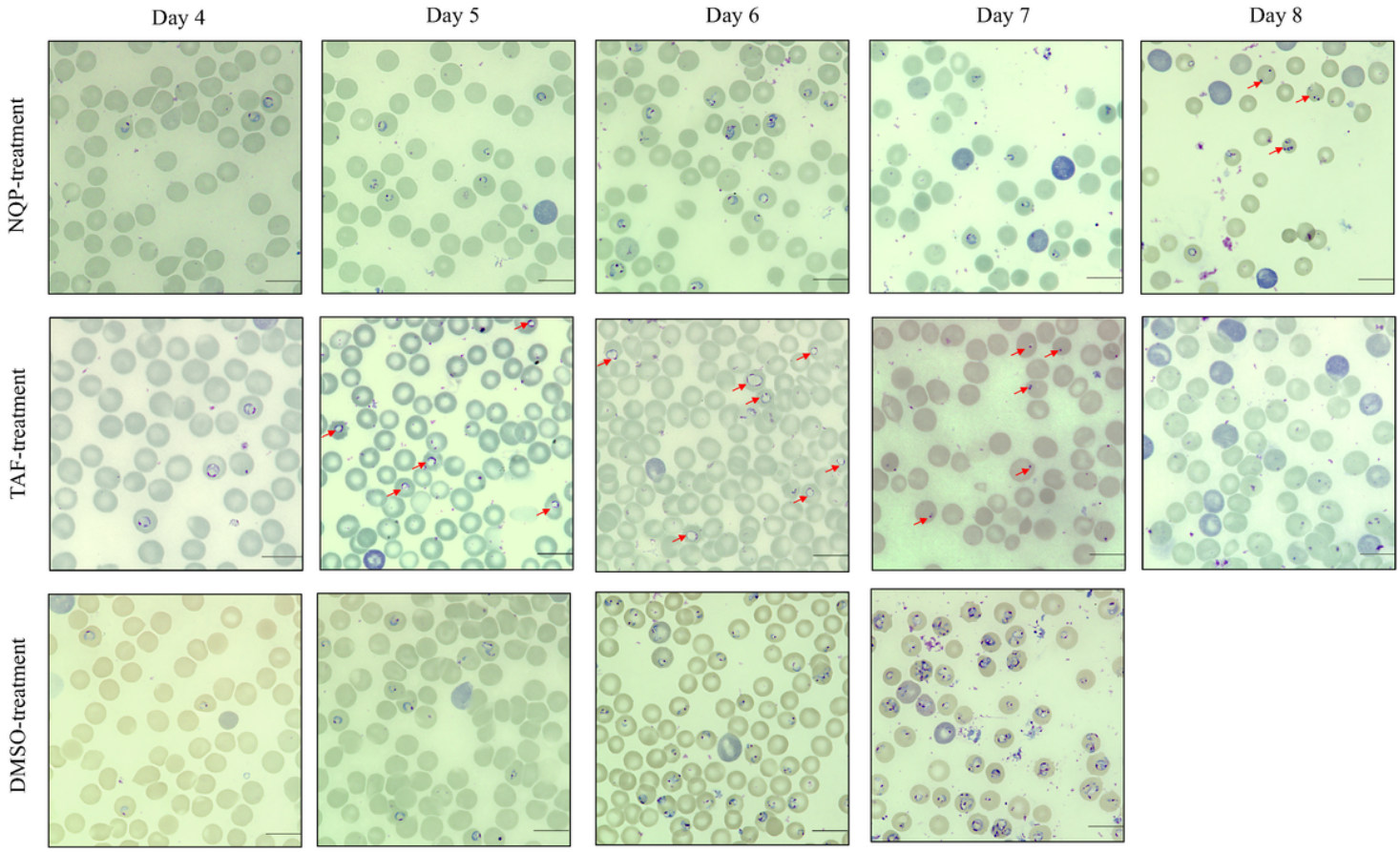

Figure 2

Light micrographs of B. rodhaini-infected mice during NQP and TAF treatment (from 4 to 8 dpi) and DMSO treatment (from 4 to 7 dpi). Compared with the DMSO-treated group, NQP treatment exhibits degenerated parasites at 8 dpi (red arrow), whereas parasites in the TAF-treated group show a vacuole-like aberrant phenotype. Bars = 10 μm.

Supplementary Files

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