Efficacy of ursolic acid against *Echinococcus granulosus* in vitro and in a murine infection model

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**Abstract**

**Background:** Cystic echinococcosis is a global public health problem; however, the drugs (albendazole and mebendazole) currently recommended by WHO for its treatment, have limited efficacy. Therefore, novel drugs are required to provide more choices for the treatment of this disease.

**Methods:** The anthelmintic effects of ursolic acid (UA) were tested on *Echinococcus granulosus* protoscoleces, germinal cells and metacestodes in vitro. The in vivo efficacy of UA was investigated in mice following secondary infection with *E. granulosus*. Furthermore, the corresponding ultrastructural damage induced by UA was evaluated by electron microscopy.

**Results:** In vitro, 45.95 ± 5.30% of protoscoleces were killed by UA at 40 \(\mu\)g/ml, while the growth of more than 90% of germinal cells was inhibited by UA at 10 to 40 \(\mu\)g/ml. The same effect was observed in metacestodes 7 days after treatment with UA at 10, 20 and 40 \(\mu\)g/ml, and more than 50% of metacestodes showed loss of integrity at the end of the experiment. In vivo, metacestode weight was significantly reduced following oral administration of UA at 200 and 100 mg/kg (39.5 and 38.3%, respectively). Additionally, ultrastructural damage, such as alternations in germinal cell morphology and formation of vacuoles and lipid granules were observed in parasites treated with UA in vitro, while detachment of the germinal layer from the laminated layer was also seen in metacestodes in vivo.

**Conclusions:** UA was demonstrated to exert parasiticidal activity against *E. granulosus* in vitro and in vivo, thus implicating UA as a potential anti-echinococcosis agent.

**Keywords:** *Echinococcus granulosus*, Ursolic acid, Protoscoleces, Germinal cells, Metacestodes, Ultrastructural damage

**Background**

Cystic echinococcosis (CE), also known as hydatid disease, which is caused by taeniid cestodes *Echinococcus granulosus* (*sensu lato*) in the larval stage, represents an important public health problem due to its wide geographical distribution as well as its medical and economic impact [1]. China is considered to be one of the most important endemic regions of this disease [2]. The life-cycle of *E. granulosus* involves dogs and other canids as definitive hosts, and numerous mammalian species, including domestic livestock and humans as intermediate hosts. Once the eggs are ingested by humans, hydatid cysts may develop, mostly in the liver and lungs. Despite its importance with regard to health, there are only two drugs (albendazole and mebendazole) available for the treatment of echinococcosis, both exhibiting some adverse effects and limited efficacy in the chronic stage of the disease [3]. Therefore, the development of novel drugs to provide more choices for the treatment of CE is necessary.

To date, several drugs that inhibit cancer cell proliferation have been evaluated for their effects on *Echinococcus* spp. based on the similarities between cancer cells and parasites. Tamoxifen, which was widely used for treating primary breast cancer, inhibited the survival of...
E. granulosus protothecoles and metacestodes at 10 to 50 μM, and a reduction in cyst weight was observed following the administration of 20 mg/kg tamoxifen [4]. The proteasome inhibitor, bortezomib, which was developed for myeloma chemotherapy, displays high activity against E. multilocularis metacestodes in vitro although the activity of this drug was lower than that of albendazole and induced adverse effects in vivo [5]. Furthermore, 5-fluorouracil and paclitaxel [6], as well as imatinib [7], exhibit profound anti-parasitic activity against E. multilocularis. These results suggest that novel candidate drugs for the treatment of CE might be found among the chemicals with antitumor effects.

In the present study, we investigated the efficacy of ursolic acid (UA) against E. granulosus. As an ursane-type pentacyclic triterpenic acid, UA is ubiquitous in the leaves and berries of natural medicinal plants [8, 9]. UA induces apoptosis and inhibits proliferation or growth in many types of malignancies both in vivo and in vitro [10–12]. UA has been developed as a liposome formulation for the treatment of cancer and the phase I trials demonstrated tolerable toxicity and adverse effects [13]. UA was also reported to have biological potential as an antibacterial [14] and antiviral [15] agent. Furthermore, the antiparasitic activity of UA has been demonstrated in protozoa [16] and helminths [17–19].

In this study, we evaluated the in vitro anthelmintic effects of UA on protothecoles, germinal cells and metacestodes of Echinococcus granulosus. Moreover, the in vivo efficacy and cytotoxicity of UA were also investigated in experimentally infected mice.

Methods

Chemicals and reagents
UA (purity > 98%) and mebendazole (purity > 98%) were purchased from Aladdin (Shanghai, China) and Sigma-Aldrich (St. Louis, USA), respectively. These powders were prepared as a 10 mg/ml stock solution in DMSO for in vitro experiments. On the day of use, the stock solutions were diluted in DMSO to the desired concentration. For in vitro experiments, these two chemicals were suspended in 1% tragacanth solution and stored at 4 °C. Unless stated otherwise, all culture media and reagents were purchased from Gibco-BRL (Zurich, Switzerland).

Parasites and animals
Liver hydatid cysts were obtained from sheep suffering from echinococcosis immediately after slaughter at abattoirs in Qinghai, China. The protothecoles from the cysts were rinsed 5–8 times with PBS containing penicillin G (500 U/ml) and streptomycin (500 U/ml) for the collection of live parasites. Female Kunming (KM) mice (aged 4 weeks, 18–22 g) were purchased from the SLAC Laboratory Animal Center (Shanghai, China). Each mouse was inoculated intraperitoneally with 2000 protothecoles. The genotype of protothecoles from sheep and germinal cells from secondary infected mice was identified as E. granulosus G1 strain [20].

In vitro culture of protothecoles, germinal cells and metacestodes
Protothecoles were maintained as previously described [20] with minor modifications. In brief, protothecoles were cultured in RPMI 1640 medium supplemented with 10% FBS, 10% hydatid fluid, reducing agents (1 × 10^−5 M L-dithiothreitol and 100 μM L-cysteine), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin G, and 100 μg/ml streptomycin, at 37 °C under 5% CO2. The culture medium was changed every 4–5 days. Metacestodes were collected aseptically from the mice more than 10 months after secondary infection with E. granulosus protothecoles. Only the cysts distributed in the abdominal cavity and not adhering to the tissues were selected for experiments. After being washed 4–5 times with PBS, metacestodes (diameter < 1 cm) were maintained using the same culture conditions as those used to maintain protothecoles. The larger metacestodes were maintained in PBS for 24 h to exclude contamination with host cells. Then germinal cells were collected and cultured as previously reported [20]. The culture conditions were the same as those used for protothecoles, although the medium was changed every 2–3 days.

Confirmation of germinal cells by qPCR
Germinal cells (without host cell contamination) were characterized by qPCR as follows: RNA was extracted from E. granulosus germinal cells, E. granulosus protothecoles (as positive control) and host tissues (normal mice liver) using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The first-strand cDNA was then synthesized from total RNA treated with DNase I (Thermo Fisher Scientific, California, USA) using the ReverTra Ace qPCR Kit (Toyobo, Osaka, Japan). PCR was performed using primers for the specific amplification of E. granulosus EF-1α [21] and mouse GADPH [22].

The qPCR reaction was performed using the C1000 Touch Thermal Cycler (Bio-rad, California, USA) in a final volume of 20 μl: 10 μl SYBR Green PCR Master Mix (Thermo Fisher Scientific), 0.8 μl of each primer (10 pmol/μl), 2 μl cDNA product, 6.4 μl dH2O. Amplification was performed using the following conditions: 95 °C for 30 s and 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s and polymerization at 72 °C for 30 s. The PCR products were then heated to 95 °C for 10 s. A melting curve was created by cooling the products to 65 °C and then heating to 95 °C at a rate
of 0.1 °C/s while measuring of the fluorescence simultaneously. PCR products were separated by 2% agarose gel electrophoresis and stained with Goodview (Beijing SBS Genetech, Beijing, China) before visualization under UV light.

The effects of UA on protoscoleces, germinal cells and metacestodes in vitro
Protoscoleces \((n = 50/\text{well})\) or germinal cells (approximately \(5 \times 10^4/\text{well}\)) were seeded in 96-well microtiter plates (Costar, Corning, USA) in 200 μl medium. Metacestodes \((n = 6/\text{well})\) were plated in 6-well microtiter plates (Costar) in 6 ml medium. UA was dissolved in DMSO and added to the medium at a final concentration of 1 to 40 μg/ml; 0.5% DMSO served as the control.

Protoscoleces and germinal cells were treated for 72 h. Metacestodes were treated by adding different concentrations of UA to the culture medium once each week for 2 weeks. Each experiment was repeated at least three times.

The viability of protoscoleces was determined using the methylene blue exclusion method as described previously [20]. In brief, 100 μl 0.1% methylene blue was added to each well. After 2 min, the protoscoleces were observed under an inverted microscope (dead protoscoleces were stained blue and the surviving ones remained colorless). The viability of germinal cells was tested using the Cell counting kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. The criteria for metacestode vitality was assessed on the basis of structural vesicle integrity.

The efficacy of UA in experimentally infected mice
KM mice \((n = 32)\) that had been infected with \(E.\ granulosus\) protoscoleces for 8 months were randomly divided into four groups (8 animals per group). These animals received UA (suspended in 1% tragacanth solution) via the oral route at 200 and 100 mg/kg/day (UA-200 and UA-100), and mebendazole (MBZ) at 25 mg/kg/day (MBZ-25) (also suspended in 1% tragacanth solution) for 28 consecutive days. The remaining group was treated orally with 1% tragacanth solution (1% TRA) as a control. During the treatment, mice were monitored daily for changes in behavior and body weight changes as well as survival. Two weeks after treatment, all mice were sacrificed by cervical dislocation, and the cysts in the peritoneal cavity were isolated and weighed. The efficacy of the treatment was assessed based on mean cyst weight [23] and ultrastructural changes in protoscoleces and germinal cells.

Morphological and ultrastructural investigations of UA-treated protoscoleces, germinal cells and metacestodes
For investigations of the ultrastructure, germinal cells were grown on glass coverslips prior to drug treatment. Samples of protoscoleces, germinal cells and metacestodes cultured in vitro and treated with UA as well as cysts obtained from treated mice and their controls were processed for evaluation by scanning and transmission electron microscopy (SEM and TEM), respectively, as previously described [24].

Cytotoxicity assay
Cytotoxicity was determined using three non-carcinogenic cell lines (1,929 cells, HK-2 cells and Chang liver cells cultured in RPMI 1640, DMEM/F12 and DMEM, respectively) and three cancer cell lines (A2058 cells cultured in RPMI1640 and A172 cells and HCT-8 cells cultured in DMEM) (all cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences); all media were supplemented with 10% FBS. The six cell types were seeded in 96-well microtiter plates \((5 \times 10^4 \text{cells/well})\). UA was added, and plates were incubated for 72 h at 37 °C under 5% CO2. Cell viability was determined with CCK-8 kits using the same method used to evaluate the viability of germinal cells. The effects of UA on non-carcinogenic cell lines were analyzed in the range 1.56–50.00 μg/ml, and on cancer cell lines in the range 0.39–12.5 μg/ml.

Statistical analysis
The differences in mean cyst weights among groups were assessed by Kruskal-Wallis H-test and paired comparisons. IC_{50} (concentration that inhibited 50% germinal cell growth) and Tox_{50} (concentration that induced 50% cell toxicity) values were calculated using the probability unit method with SPSS version 17.0; \(P < 0.05\) was considered to indicate statistical significance.

Results
The in vitro activity of UA against \(E.\ granulosus\) protoscoleces
After UA treatment at 40 μg/ml for 3 days, 45.95 ± 5.30% of protoscoleces were killed, while there was no significant effect on their viability at 20 μg/ml (Fig. 1a). Dead protoscoleces were distinguished from viable protoscoleces by the methylene blue exclusion method and visualized under light microscopy (Fig. 1b-g). The dead protoscoleces induced by UA treatment showed morphological changes, while micro-cysts were observed in the surviving protoscoleces. In contrast, no morphological changes were observed in the protoscoleces in the control group.

Ultrastructural damage was revealed by TEM in the protoscoleces treated with UA at 40 μg/ml for 3 days compared with the control protoscoleces cultured in 0.5% DMSO medium (which resulted in a death-rate of 6.43 ± 1.00%). No changes in ultrastructure were observed throughout the experimental period in the
control group (Fig. 1d, e). In contrast, the soma tegument of protoscoleces appeared swollen with partial collapse and disruption of the inner structure following UA treatment. The distal cytoplasm at the rostellar and soma tegument was almost detached from its underlying basal matrix. Moreover, partial lysis of muscle cells and extracellular matrices accompanied by the formation of large vacuoles and lipid granules were also observed (Fig. 1h, i).

The in vitro activity of UA against *E. granulosus* germinal cells

In the qPCR analysis, there was no amplification of mouse GADPH in *E. granulosus* germinal cell culture (Ct = 24.03 ± 0.23), thus confirming the absence of host cells (Fig. 2a, Lane 6), while the *E. granulosus* EF-1α specific gene was amplified only from germinal cell culture (Ct = 28.00 ± 0.11) and protoscoleces (Ct = 24.13 ± 0.62).

Compared to the effects on *E. granulosus* protoscoleces, UA had a considerably stronger, and also dose-dependent, effect on germinal cells over 3 days. At 10, 20 and 40 μg/ml, UA resulted in a dramatic reduction of viable cells, with the inhibition rate higher than 90% (Fig. 2b). Moreover, germinal cell growth inhibition was reduced to 64.68 ± 11.22% by incubation with 8 μg/ml of UA and 3.76 ± 16.53% at 4 μg/ml (Fig. 2b) and the IC50 was calculated to be 5.65 ± 0.84 μg/ml. The effects of UA on *E. granulosus* germinal cells were also confirmed at the ultrastructural level by SEM (Fig. 2c-f). Germinal cells comprise a mixture of several cell types, including undifferentiated cells, muscle cells and glycogen storage cells. Most of germinal cells were round and adhered to the coverslips, exhibiting no ultrastructural alterations during the entire incubation period in comparison to the cells in the control group (Fig. 2c, d). In contrast, ultrastructural damage was detected in 40 μg/ml UA-treated germinal cells, which exhibited dramatic changes in morphology and the almost complete loss of protrusions and adherence filaments (Fig. 2e). Moreover, aggregation of dead cells was observed (Fig. 2f).
The in vitro activity of UA against *E. granulosus* metacestodes

Untreated *E. granulosus* metacestodes obtained from in vitro cultures are basically fluid-filled cysts, with a distinct acellular outer laminated layer and an intact germinal layer. No alterations in *E. granulosus* metacestodes were observed after 14 days of incubation with 0.5% DMSO (Fig. 3a). However, the effect was detected in metacestodes treated with 10, 20 and 40 μg/ml of UA at 7 days after initiation of treatment. At the end of the experiment, more than 50% of metacestodes showed loss of integrity and typical changes, such as softening of the cysts and visible separation of the germinal and laminated layers (Fig. 3b). However, no effects on the morphology of metacestodes were observed following treatment with UA at 1 and 5 μg/ml for 14 days (Table 1).

SEM studies revealed that the germinal layer of UA-treated metacestodes lost its characteristic multicellular structure. Metacestodes treated with 0.5% DMSO exhibited an intact germinal layer with an abundance of cells (Fig. 3c), while the UA-treated germinal layer was largely detached in many areas, with only cellular debris remaining (Fig. 3d). TEM studies were conducted to provide more detailed information of the ultrastructure of metacestodes. Normally, the external surface of the parasite is surrounded by an acellular laminated layer and the parasite tissue attaches to the interior surface of the laminated layer via numerous microtriches. Furthermore, the tegument layer is located toward the interior followed by the germinal layer that contains numerous cell types. In this study, metacestodes treated with 0.5% DMSO for 14 days did not exhibit any ultrastructural
changes, while UA treatment induced drastic destruction of parasites (Fig. 3e, f) and complete separation of the germinal layer from the laminated layer. In detail, the microtriches disappeared, the tegument layer was swollen, vacuolization increased and lipid droplets formed in the germinal layer (Fig. 3f).

In the in vivo efficacy of UA in experimentally infected mice

Intraperitoneally infected KM strain mice were exposed to different treatment regimens involving MBZ and UA for 4 weeks as indicated in Table 2. At the end of treatment, these animals were sacrificed by cervical dislocation and the total parasite weight was measured to calculate the reduction in cyst weight (Table 2). There was a significant difference among the four groups ($\chi^2 = 17.769, df = 3, P < 0.001$). Furthermore, daily oral administration of MBZ at 25 mg/kg significantly decreased the cyst weight (62.9%; $P < 0.001$). Oral administration of UA at 200 and 100 mg/kg also reduced the growth of the metacestodes in vivo (39.5%, $P = 0.023$ and 38.3%, $P = 0.016$, respectively). Furthermore, the cyst weight was significantly reduced in the MBZ-25 group compared with that in the UA-200 ($P = 0.044$) and UA-100 ($P = 0.045$) groups, although there was no significant difference between the UA-200 and UA-100 groups ($P = 0.940$).

TEM and SEM studies of metacestodes isolate from mice following treatment with UA revealed extensive parasite tissue damage (Fig. 4). Hydatid cysts from infected mice treated with 1% TRA presented intact structural features, such as a well-defined laminated layer, an intact and densely-packed germinal layer, protrusion of the microtriches into the laminated layer, and abundant undifferentiated cells and muscle cells (Fig. 4a, b). SEM studies revealed distinct changes in metacestodes from MBZ- and UA-treated mice, including detachment of the germinal layer from the laminated layer (Fig. 4e) and loss of the normal structure of germinal layer (Fig. 4c, g). Following treatment, TEM studies showed marked destruction of the germinal layer characterized by the absence of cellular structures, together with the formation of vacuoles in the tegument and swelling or destruction of microtriches that were partially separated from the laminated layer (Fig. 4c–h).

Cytotoxicity of UA in non-carcinogenic cells and cancer cell lines

The cytotoxicity of UA was assessed in mammalian non-carcinogenic cell lines (L929, HK-2 and Chang liver) and cancer cell lines (A172, A2058 and HCT-8) using the CCK-8 method. The Tox50 values for the three non-carcinogenic cell lines were 13.69 ± 1.39, 11.41 ± 2.24 and 13.97 ± 1.67 μg/ml, respectively. In contrast, the cancer cells were more sensitive to UA, with Tox50 values of 2.54 ± 0.20, 2.68 ± 0.04 and 2.80 ± 0.02 μg/ml, respectively.

Discussion

Since drug research and development is time-consuming and expensive, a drug repurposing strategy offers an alternative route to significantly shorten the traditional...
drug development process [25]. In the present study, we investigated the in vitro anthelmintic effects of UA on protoscoleces, germinal cells and metacestodes of *E. granulosus* and the in vivo efficacy in experimentally infected mice. Although treatment with 40 μg/ml UA for 3 days only resulted in the death of 45.95 ± 5.30% protoscoleces, the survival of germinal cells was found to be inhibited in a dose-dependent manner, with > 90% inhibition at 10, 20 and 40 μg/ml. These results indicated that germinal cells were more sensitive to UA than protoscoleces. This information has important implication for the potential treatment of echinococcosis, because germinal cells are the only proliferative part of the parasite and are usually considered as the main target for chemotherapy [26]. In addition, UA was also shown to be effective on cultured metacestodes in vitro. In the infected organs (especially the liver and lungs) of hosts, these cysts have a basic bladder-like morphology and are protected by the extracellular carbohydrate-rich laminated layer (LL), which is synthesized by the underlying cellular germinal layer (GL) [27]. Our results confirmed the ability of UA at effective concentrations to cross the LL to induce the death of the parasites. In our in vitro study, MBZ was used as the positive control and 0.5% DMSO was used as the negative control since this is the final concentration of DMSO required for complete dissolution of MBZ. The final results indicated that the negative control did not influence the activity of parasites during the observation period.

To test the in vivo efficacy, UA was administered at 200 and 100 mg/kg for the treatment of echinococcosis according to the regimen adopted for the treatment of cancers with UA [11, 28]. After treatment for 28 days, reduction in growth and cyst weight as well as loss of metacestode integrity was found, and this effect was significant in the groups treated with 200 mg/kg and 100 mg/kg of UA. Furthermore, the toxicity of UA was also discussed in our study. To properly compare the parasite and mammalian cells, the same viability assay was applied by CCK-8 kit. For three mammalian normal cell lines, the Tox50 was calculated to be 11–14 μg/ml, which was 2- to 3-fold in excess of the measured IC50 values observed for germinal cells. These results proved that UA was more effective against parasites than normal host cells, but also indicated a potential safety concern. Hence, during the 28 days of treatment, we paid close attention to the conditions of the animals. However, no mice died during the experiments, while two mice and one mouse died in the control and MBZ-25 groups, respectively, which preliminarily indicated

### Table 2 Effects of UA on mice infected with *E. granulosus* for 8 months (n = 8)

| Group                  | Dose (× 28 days) | Mean cyst weight ± SD (g) | Mouse deaths during the experimental period | Cyst weight reduction (%) |
|------------------------|-----------------|---------------------------|--------------------------------------------|---------------------------|
| Control (1% tragacanth) | 0.1 ml/10 g/day | 19.73 ± 17.22             | 2                                          | –                         |
| Mebendazole            | 25 mg/kg/day    | 7.33 ± 3.67*              | 1                                          | 62.9                      |
| Ursolic acid 200 mg/kg/day | 11.94 ± 5.69*       | 1                          | 39.5                                      |
| 100 mg/kg/day          |                 | 12.18 ± 8.64*             | 0                                          | 38.3                      |

*Indicates that there were significant reductions in parasite weight achieved by treatment with mebendazole at 25 mg/kg and UA at 200 mg/kg and 100 mg/kg compared that achieved by treatment with 1% tragacanth

Abbreviation: SD, standard deviation
that the use of UA for the treatment of murine-echinococcosis was safe in our conditions. In addition, the regimen used in this study has been proved to be safe in the treatment of parasites and cancer in animals and humans [8, 18]. Still, considering long-term chemotherapy for echinococcosis, scientific and objective safety and toxicity of UA must be evaluated in future.

The efficacy of UA against echinococcosis in the murine model might be attributed to its direct effect on the parasite, as well as its impact on host according to the mechanisms of UA against cancers. It was reported that UA can inhibit cell growth, migration and invasion in many cancer cell models and exert antiproliferative effects [8] via several mechanisms that are also important for the development and survival of Echinococcus spp. For example, UA was reported to induce apoptosis through suppression of the components of the ERK/MAPK signaling pathway [29, 30], which plays important roles in the growth and development of Echinococcus spp. [31, 32]. Human tumor suppressor p53 was effected by UA, and its homologue-Emp53 in E. multilocularis was reported to play a functional role in the E. multilocularis metacestode [33]. In addition to the induction of apoptosis, UA has also been reported to prevent the metastasis of cancer through down-expression of matrix metalloproteinases (MMP-2 and MMP-9) [12, 34] relating to the microenvironment of cancer cells; these enzymes were detected in E. granulosus hydatid cyst fluid, cyst membranes and protoscolices, and are involved in pathology-related tissue remodeling [35, 36].

Poor solubility of UA in aqueous solutions greatly limits its application [13]. In the treatment of several cancers, no differences of efficacy were shown between 50 mg/kg/d and 200 mg/kg/d of UA [37, 38]. In our study, similar therapeutic outcomes of the two UA groups were also found. Hence, because of its poor water solubility and low bioavailability, UA has been administered using liposomes [39, 40]. This characteristic of UA indicates that the appropriate formulation of UA could potentially enhance the treatment efficacy against echinococcosis.

Conclusions

The findings of the present study demonstrate the efficacy and parasiticidal activity of UA against E. granulosus protoscoleces, germinal cells and metacestodes in vitro and in vivo. Thus, UA is implicated as a potential anti-echinococcosis agent. However, its mechanism and the application prospects with proper formulation remain to be investigated.

Abbreviations

CCK-8: cell counting kit-8; CE: Cystic echinococcosis; GL: Geriminal layer; KM mouse: Kunming mouse; LL: Laminated layer; MBZ: Mebendazole; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy; UA: Ursolic acid; WHO: World Health Organization

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Authors’ contributions

JC and JY conceived and designed the study. JY and CL performed the experiments and data analysis. JC, YS and HZ contributed reagents and materials. JY wrote the paper and JC revised the paper. All authors read and approved the final manuscript.

Ethics approval

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology, China. The protocol was approved by the Laboratory Animals Welfare and Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (permit number: PD-2014-2). A special effort was made to reduce the number of mice used in the study, to provide them with the most comfortable conditions, and to minimize animal suffering wherever possible.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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