

In vivo evaluation of the efficacy of combined albedazole-IFN- α treatment for cystic echinococcosis in mice

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Abstract Cystic echinococcosis (CE) is a serious helminthic zoonosis caused by *Echinococcus granulosus* metacestode worldwide. The current chemotherapy of CE is mainly based on albendazole (ABZ). However, more than 20% CE cases failed to such chemotherapy. Thus, novel and more efficient treatment options are urgently needed. This study was to evaluate the in vivo efficacy of combined ABZ-interferon (IFN)- α treatment for CE in mice. After 5 months of secondary infection with protoscoleces, mice were randomly allocated into four groups: ABZ-treated group, IFN- α -treated group, ABZ+IFN- α group, and untreated control group. Drugs in diverse treated groups were respectively administered for 2 months. Mice were then euthanized and associated indications were investigated to evaluate the therapeutic efficacy. ABZ+IFN- α induced a significant reduction of the number, size, as well as weight of cysts, compared with that in the ABZ ($p < 0.05$) or untreated group ($p < 0.01$), respectively. This effect was associated with ultrastructural modification of the cyst in the ABZ+IFN- α group. Interestingly, significant decrease of IL (interleukin)-10 in serum and in vitro production by spleen cells with ABZ+

IFN- α treatment was observed in comparison with untreated control ($p < 0.01$). Serum IgE, IgG, and subsets were respectively decreased in ABZ+IFN- α treatment, compared with that in the control group ($p < 0.01$). In conclusion, our findings demonstrated that combination of ABZ with IFN- α may contribute to an efficient therapeutic regimen of human and animal CE.

Keyword Cystic echinococcosis (CE) · Albendazole · IFN- α · IL-10 · Ultrastructural modification

Introduction

Cystic echinococcosis (CE) is a serious helminthic zoonosis caused by *Echinococcus granulosus* metacestode worldwide, which causes considerable economic losses and contributes a threat to public health in many countries (Brunetti et al. 2010). Treatment of CE routinely relies on surgery and/or chemotherapy, depending on different factors such as cyst size and location, viability, microbial infection, and potential dangerous complications related to cyst rupture (Ceballos et al. 2008; Kern 2006; Menezes da Silva 2003). Surgery, the predominant strategy to treat CE, has limitations such as recurrence of cysts and contraindications (inactive asymptomatic cysts, difficult to access cysts, and very small cysts) (Brunetti et al. 2010). As one of the complements for surgery, the only drugs available for the treatment of CE are benzimidazole carbamate derivatives, namely mebendazole (MBZ) and albendazole (ABZ) (Falagas and Bliziotis 2007). However, more than 20% CE cases failed to such chemotherapy (Moro and Schantz 2009). Thus, novel and more efficient treatment options are urgently needed.

The search for novel therapeutic options to treat CE is ongoing, and numerous studies found a handful of potential

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candidates available for CE, including 5-fluorouracil (FU) and paclitaxel (PTX) (Pensel et al. 2014), ABZ sulfoxide-loaded solid lipid nanoparticles (Ahmadnia et al. 2013), combined flubendazole-nitazoxanide (Laura et al. 2015), *Zataria multiflora* methanolic extract (Moazeni et al. 2014), artemisinin and artemisinin derivatives (Spicher et al. 2008), thymol (Maggiore et al. 2015), biogenic selenium nanoparticles (Mahmoudvand et al. 2014), praziquantel (Gavidia et al. 2010; Jamshidi et al. 2008), and lufenuron (Breijo et al. 2011). However, no ideal therapeutic regimen for clinical application in CE.

IFN- α , one member of type I interferon (IFN) family, primarily used as anti-virus therapy for various viral disease (Uze and Tavernier 2015). Subsequently, IFN- α has been shown to exhibit multiple biological functions. The mainly clinical use of IFN- α is treated for viral infectious disease, neoplastic disease and tumors (Gibbert et al. 2013; Uze and Tavernier 2015). For another Echinococcus infectious disease-AE, several studies have revealed that IFN- α may induce protective effects. Godot V et al. demonstrated that the lesion size from AE patients were regressed treated by alone IFN- α (Godot et al. 2003). IFN- α treatment also could reverse Th2 to Th1 immune response in AE. Indeed, Th2 humoral response contributes parasite evasion during chronic echinococcosis, whereas Th1-induced cellular immunity help hosts to remove the parasite. Thus, IFN- α treatment may contribute to host protection by inhibiting Th2 reaction. Moreover, IFN- α regressed the liver fibrosis and lesion. However, there is lack of investigation to IFN- α treatment for CE.

The aim of the study was to evaluate the in vivo efficacy of alone IFN- α and combined ABZ-IFN- α treatment for CE in mice. And our results found that combined ABZ-IFN- α treatment showed more effective for CE than treated with ABZ or IFN- α .

Materials and methods

Animals and drugs

All animal experiments were approved by the Ethics Committee of Ningxia Medical University and carried out in accordance with the 2011 revised form of The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Forty female Balb/c mice (8 weeks old at the start of the experiments) were obtained from Vital River Laboratory Animal Technology Co. Ltd., Beijing, China. The animals were housed in a temperature-controlled (22 ± 1 °C), light-cycled (12-h light/dark cycle) room.

ABZ was purchased from GSK (GlaxoSmithKline, UK); Pegasys® interferon alfa-2a was obtained from Roche.

Protoscoleces collection

The hydatid cysts were obtained from the liver of a single CE patient in the clinical operation and aseptically transferred to the laboratory. Vesicle fluid were obtained by puncturing, and then separated by centrifugation at $9000 \times g$ for 20 min at 4 °C. The parasites were washed twice using phosphate-buffered saline (PBS) (pH 7.2, containing 1000 µg/mL penicillin and 1000 U/mL streptomycin (Invitrogen, MD, USA)). The parasite vitality was determined by methylene blue exclusion test (Elisondo et al. 2006). Only parasite batches exhibiting over 90% vitality were used. The final concentration was 1500 live protoscoleces in 200-µL PBS.

Experimental design

Female Balb/c mice ($n = 40$; body weight, 25 ± 5 g) were infected by intraperitoneal (i.p.) inoculation of fresh 1500 protoscoleces/animal, suspended in 200-µL PBS. After 5-month postinfection (p.i.), mice were randomly allocated into four groups (10 animals/group): (a) ABZ-treated group, animals were treated with the suspension of ABZ (5 mg/kg) by the intragastric administration; (b) IFN- α -treated group, animal received an intramuscular injection of IFN- α -2a 10,000 IU/50 µL every 3 days; (c) ABZ+IFN- α group, animals were treated with both ABZ and IFN- α -2a as described in a and b; and (d) untreated control group, animals received distilled water as a placebo. Drugs in diverse treated groups were respectively administered for 2 months. Mice were then euthanized and associated indications were investigated to evaluate the therapeutic efficacy.

Determination of the parasite burden and efficacy rate of treatments

Cysts were obtained from the peritoneal cavity of sacrificed mice. The number, size, as well as the weight of cysts from each animal were measured. The efficacy of treatments (based on the weight of cysts from infected mice) was calculated by the use of the following formula (Pensel et al. 2015):

$$\text{Efficacy} = \frac{X_c - X_t}{X_c} \times 100\%,$$

where X_c is the mean cyst weight in the untreated control group and X_t is the mean cyst weight in the treated group.

Transmission electron microscopy

Samples of cysts from each group were processed for TEM as described previously (Ahmadnia et al. 2013). The cysts were sectioned to blocks of about 1 mm^3 in dimensions and fixed 5 h in cold 2.5% glutaraldehyde diluted in 0.1 M PBS. The

samples were washed twice in 0.1 M PBS and then fixed in 1% osmium tetroxide. They were then dehydrated in a graded series of ethanol and subsequently embedded in an equal volume of propylene oxide and Epon resin overnight and then in fresh 100% Epon for another 24 h. Finally, the blocks were polymerized in fresh Epon resin by leaving them in an oven at 65 °C for 18 h. Using an ultramicrotome (Leica EM FC7, Germany), sections of 50–70 nm in thickness were provided and were loaded on 200-mesh copper grids having carbon-stabilized formvar support films. The sections were then stained by immersion in saturated uranyl acetate for 10 min and lead citrate for 5 min. They were finally washed by distilled water, air dried, and examined with an electron microscope (H7650, HITACHI, Japan).

ELISA assays

Spleen cells were isolated from each individual for in vitro assays. In the presence of 2.5 µg/mL of Con-A (Sigma), 5×10^6 splenocytes per milliliter were incubated at 37 °C with 5% CO₂ in RPMI 1640 (Gibco BRL) containing 10% heat inactivated fetal calf serum (FCS). Supernatants were collected after 72 h for determination of IL-4, IL-10, and IFN-γ concentrations. Measurement of each cytokine production in serum and supernatants was performed by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (e-Bioscience, CA, USA).

Serum antibody levels were also detected by ELISA after mice sacrifice. Ninety-six-well microplates (Sino-American Biotechnology Company, Beijing, China) were coated with 100-µL (0.1 µg/µL) crude antigens from cysts per well and incubated overnight in 0.1 M carbonate buffer (pH 9.6) at 4 °C. Serum samples were diluted (1:100) in PBST buffer (pH 7.2, PBS with 0.05% Tween-20) and incubated at 37 °C for 1 h in duplicate. Then, bound antibody were detected by HRP-conjugated goat anti-mouse IgG, IgG subclass, IgE, and IgM (Novagen) at 1:1000 dilution in PBST buffer. Optical density (OD) values were read at 490 nm (Bio-Rad).

Statistical analysis

All data was analyzed using Prism 5.0 (GraphPad Software Inc., CA, USA). The results were represented as

Table 1 The number of hydatid cysts in control and treated infected mice

Groups	No. of cysts in individual mice										Mean ± SEM
ABZ	3	2	2	1	4	1	2	1	3	2	2.100 ± 0.314
IFN-α	4	7	3	4	5	1	6	2	4	2	3.800 ± 0.592
ABZ+IFN-α	1	1	1	0	2	1	1	2	0	0	0.900 ± 0.233** [#]
Untreated control	5	4	0	6	2	3	3	5	7	4	3.900 ± 0.640

Note: The number of cysts in ABZ+IFN-α-treated group showed significant reduction from group untreated control (** $p < 0.01$) or group ABZ ([#] $p < 0.05$)

mean ± standard error (SD), and one-way analysis of variance were used to compare data displaying a normal distribution and homogeneity of variance. Parasite weights and size were compared using one-way ANOVA testing. Cytokine concentrations and antibody levels were compared by one-way ANOVA analysis. Independent *t* test was used for comparison of two groups. Results were considered significant at $p < 0.05$.

Results

ABZ+IFN-α treatment reduced cyst burden

None of the animals died in the experiments, indicating no serious side effect of drugs. The mean body weight in four groups showed no significant difference ($p > 0.05$, data not shown). The number of cysts in the ABZ+IFN-α-treated group showed significant reduction from the group untreated control ($p < 0.01$) or group ABZ ($p < 0.05$) (Table 1). Cyst counts in the ABZ-treated group were decreased, compared with that in the untreated control group ($p < 0.01$). However, no significant reduction in cyst burden was observed between the IFN-α-treated- and untreated group ($p > 0.05$).

Similarly, our results showed that either cyst weights or size in the ABZ+IFN-α-treated group were significantly reduced, compared with the untreated control group ($p < 0.01$) or ABZ group ($p < 0.05$) (Table 2). The efficacy rate in the ABZ+IFN-α-treated group is up to 89.7% (Table 2). These results indicated that ABZ+IFN-α treatment induced a reduction of cyst burden.

Table 2 Weights and size of hydatid cysts in control and treated infected mice

Groups	Cyst weights (g)	Cyst diameter/mice (mm)	Efficacy rate (%)
ABZ	0.21 ± 0.17**	2.1 ± 0.9**	78.4
IFN-α	0.89 ± 0.28	6.7 ± 1.5	0.08
ABZ+IFN-α	0.10 ± 0.05** [#]	1.2 ± 0.4** [#]	89.7
Untreated control	0.97 ± 0.45	7.3 ± 3.2	—

Note: Weights and size of cysts in ABZ+IFN-α-treated group showed significant reduction from group untreated control (** $p < 0.01$) or group ABZ ([#] $p < 0.05$)

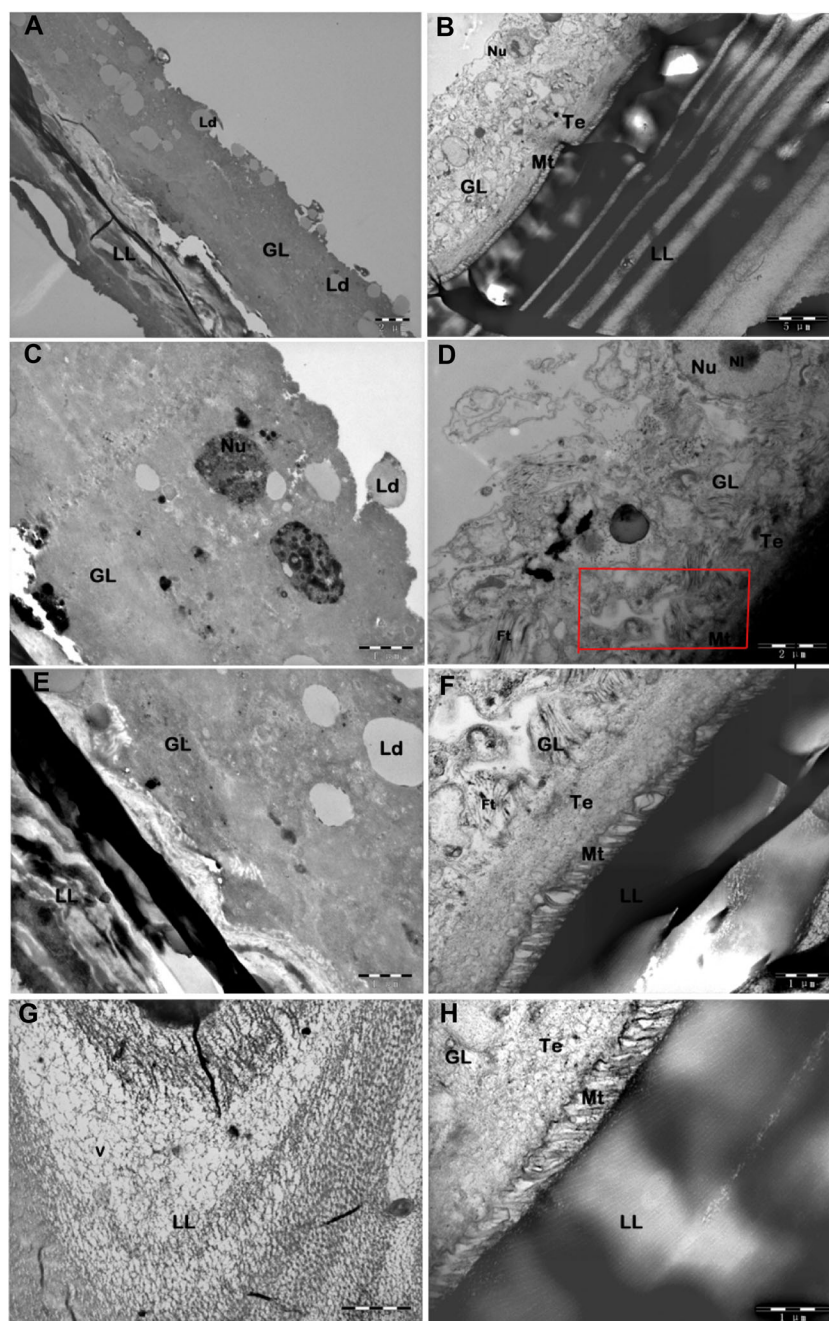


Fig. 1 Ultrastructural modification of cyst wall and cyst components in ABZ+IFN- α -treated group. ABZ+IFN- α -treated group (**a**, **c**, **e**, and **g**) and untreated group (**b**, **d**, **f**, and **h**). **a** and **b** showed the general ultrastructure of cysts. **c** and **d** showed germinal layer of cysts. **e** and **f** emphasized on changes of teguments and microtriches. **f** derived from *red rectangle area* of **d**. **g** and **h** represented laminated layer of cysts. In untreated group, the hydatid cyst appears almost normal laminated layer

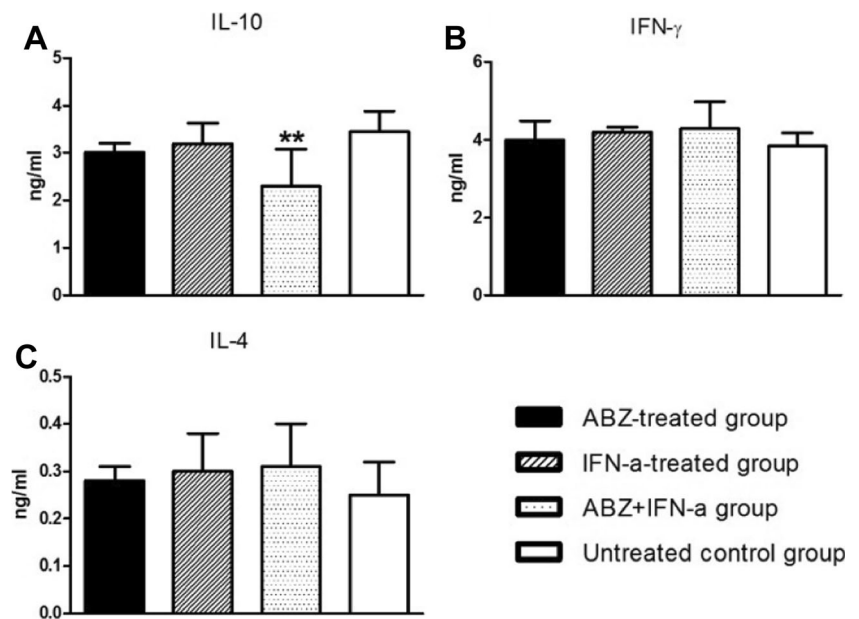
(LL), germinal layer (GL), tegument (Te), microtriches (Mt), undifferentiated cells (UC), fibrous tissue (F), cell nucleus (Nu), and nucleolus (NI) are clearly visible. In ABZ+IFN- α -treated group, ultrastructural alternations shows as lipid droplets (Ld) and vacuoles (V), nuclear fragmentation, degeneration, and disappearance of microtriches (Mt), undifferentiated cells (UC), tegument (Te), and cellular elements. Each figure was labeled by a *scale bar*

The cysts in the ABZ+IFN- α -treated group showed more ultrastructural modification

TEM was performed to evaluate the effects among treatments on ultrastructural modification of cysts. The untreated control

group exhibited the typical structure of *E. granulosus* cyst, whereas the ABZ+IFN- α -treated group showed ultrastructural alteration. The general ultrastructure of cyst in the ABZ+IFN- α -treated group was greatly changed (Fig. 1a) in comparison of that in normal control (Fig. 1b). In germinal layer,

Fig. 2 Significant decrease of IL-10 in serum with ABZ+IFN- α treatment. Sera of mice from diverse groups were collected respectively for detection of IFN- γ (a), IL-4 (b), and IL-10 (c) concentration using ELISA kit. $**p < 0.01$



normal undifferentiated cells, fibrous tissue, and classical nucleus and nucleolus were disappeared, instead, nuclear fragmentation and numerous lipid droplets were found in the ABZ+IFN- α -treated group (Fig. 1c, d), indicating generation of daughter cysts by germinal layer was destroyed. Typical tegument and regular microtriches disappeared in the ABZ+IFN- α -treated group (Fig. 1e), compared with that in the control group (Fig. 1f). Normal outer surface of cyst was formed by the acellular, carbohydrate-rich laminated layer in regular arrangement, which also was replaced in the ABZ+IFN- α -treated group by irregular and distorted structure rich in vacuoles. (Fig. 1g, h). The ABZ-treated group showed similar

ultrastructural modification with the ABZ+IFN- α -treated group (data not shown). Taken together, structural alterations of the cysts in the ABZ+IFN- α -treated group were greatly changed and showed morphological heterogeneity.

Significant decrease of IL-10 in serum with ABZ+IFN- α treatment

To assess cytokine status after treatment, we found decreased IL-10 level in serum from the ABZ+IFN- α group, compared with that in the control group (2.29 ± 0.25 vs. 3.44 ± 0.14 ng/ml,

Fig. 3 Significant decrease of IL-10 in production in vitro with ABZ+IFN- α treatment. Cytokine production by spleen cells from diverse groups was collected respectively for detection of IFN- γ (a), IL-4 (b), and IL-10 (c) secretion using ELISA kit. $**p < 0.01$

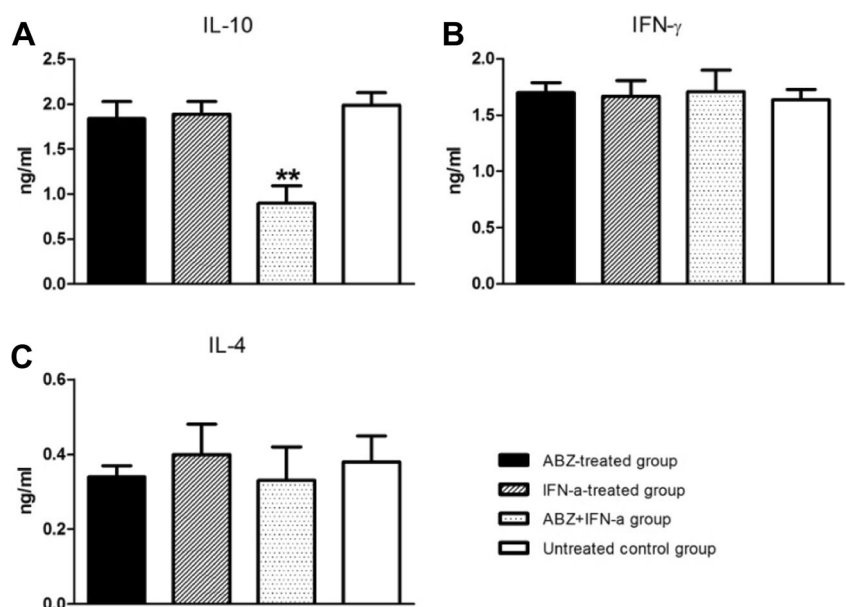
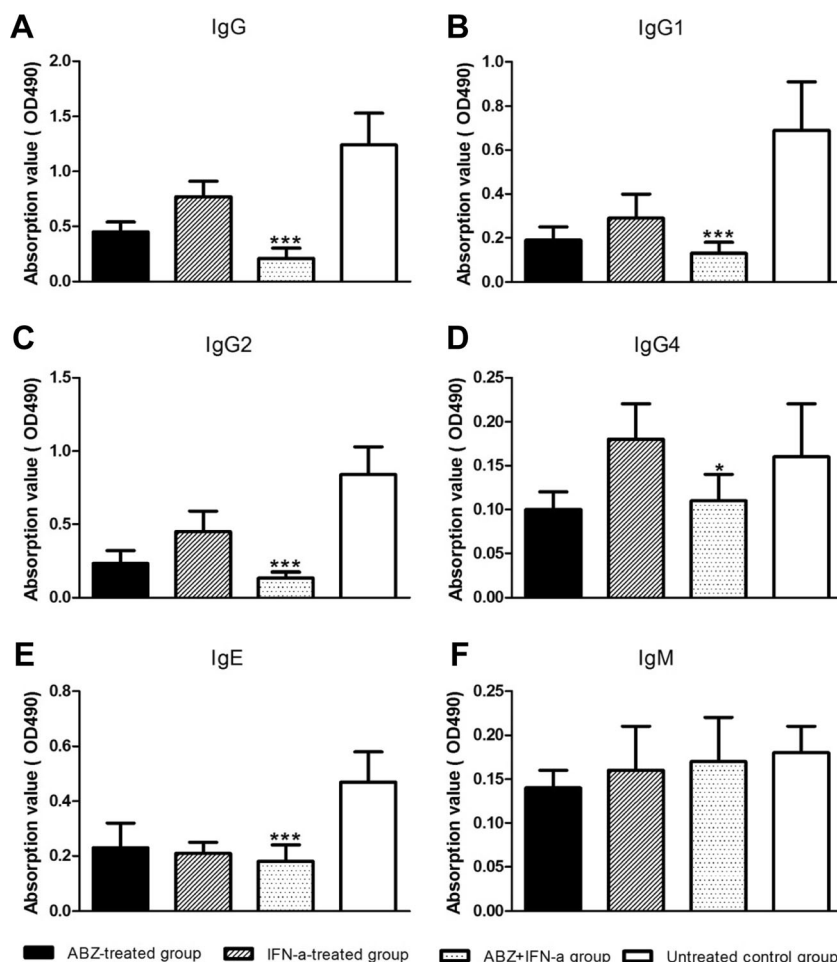


Fig. 4 Detection of OD value profiles of IgG, IgG1, IgG2, IgG4, IgE, and IgM in diverse treated groups. The assay for antibody analysis was repeated five times. Values represent the mean $\pm$ S.D. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$



$p < 0.01$) (Fig. 2a). It showed no significant difference in IFN- γ (Fig. 2b, $p = 0.08$) and IL-4 (Fig. 2c, $p = 0.11$) levels.

Significant decrease of IL-10 in production in vitro with ABZ+IFN- α treatment

Similarly, we detected the cytokine production by spleen cells and found that IL-10 secretion in the ABZ+IFN- α group was decreased in comparison with that in the control group (0.91 ± 0.10 vs. 1.99 ± 0.17 ng/ml, $p < 0.01$) (Fig. 3a). It showed no significant difference in IFN- γ (Fig. 3b, $p = 0.31$) and IL-4 (Fig. 3c, $p = 0.18$) levels.

Serum specific humoral immunity were decreased in ABZ+IFN- α treatment

To understand the level of humoral immune response after our combinational treatment, serum antibody levels were detected by ELISA. IgG level was significantly decreased, compared with that in the untreated control group ($p < 0.001$, Fig. 4a). This decrease was primarily due to a reduction in IgG1

($p < 0.0001$, Fig. 4b), IgG2 ($p < 0.0001$, Fig. 4c), and IgG4 ($p = 0.0299$, Fig. 4d). IgE showed significant decrease between the ABZ+IFN- α -treated group and control group ($p < 0.0001$, Fig. 4e), while IgM had no difference ($p = 0.5942$, Fig. 4f).

Discussion

In the present study, we firstly investigated the efficacy of combined ABZ+IFN- α treatment for CE. By in vivo treatment of CE in mice, our study demonstrated that combination of ABZ with IFN- α showed more effective in reduction of cyst burden, suggesting that this combination exhibited therapeutic potential. Our further study revealed that this effective treatment may be associated with more ultrastructure alteration and the decrease of IL-10 level.

The crucial role of IFN- α in the reversal of a Th2 type cells to Th1 phenotype has been previously reported (Romagnani 1995). To our knowledge, the experimental and clinical

studies of the effects of IFN- α on helminth infection are limited. But it is worth mentioning in alveolar echinococcosis (AE), IFN- α regressed lesion size in human and mouse (Godot et al. 2003; Harraga et al. 1999). These therapeutic effects are due to a reversal of a Th2 to Th1 cytokine profile by downregulating IL-10, IL-6 as well as IL-13 and upregulating IFN- γ level (Godot et al. 2003). But our results demonstrated that alone IFN- α showed no capacity to regress cyst burden of CE, without significant difference of Th cytokine profile, demonstrating that alone IFN- α could not break out Th2 immune response generated by the parasite in chronic CE. This result was not paralleled with that in AE reported previously. Interestingly, combination of IFN- α with ABZ induced a significant reduction of cyst burden than the control or single ABZ group. With eradication by ABZ administration, IFN- α could inhibit Th2 by reduction of abnormal elevated IL-10, which as one of Th2 indicators impaired the Th1 protective response and help the parasite evasion in the host (Amri et al. 2009).

Humoral immune response plays a regulatory role in chronic CE as described before (Rigano et al. 1995; Zhang et al. 2012). In the present study, IgG, IgG1, IgG2, IgG4, and IgE levels significantly decreased in combinational treatment, compared with that in the untreated control group. This finding indicated that abnormal elevated humoral immune response was reduced during eradication by ABZ and IFN- α treatment. Although IgG levels were decreased, we found the level still high than the negative control in ELISA. The positive level of antibody in the treated group may be associated with immune memory. This issue needs to be further investigated. IgM, an indicator for early infection, showed no difference between diverse treatments.

In this study, IFN- γ in the ABZ+IFN- α or single IFN- α group showed no significant increase, which was consistent with IFN- α treatment for AE in mice (Godot et al. 2003). This negative result may be due to a transient increase at the beginning of the infection or treatment. IFN- γ production in viral and bacterial infection in mice reached a maximum of 7–8 days after the beginning of infection and declined thereafter (Gessner et al. 1989). In this regard, indeed, we are going to investigate early IFN- γ level in the murine experiments. Besides, due to the controversial effects of IFN- α in various diseases, our results demonstrated that IFN- α showed no direct treatment for protoscoleces of *E. granulosus* (data not shown) in vitro; the role of IFN- α in human CE should be investigated in further clinical research. Over the last decade, numerous studies reported IFN- α can act as an effective antiviral and antitumor vaccine adjuvant by enhancing dendritic cells (DC) (Arico and Belardelli 2012; Rizza et al. 2011). But whether DC participates in IFN- α treatment in CE are needed for further research.

In summary, this study firstly elucidated that combination of ABZ with IFN- α exhibited more effective for therapeutic

regimen in CE than alone treatment and study on the above issues of this treatment is currently ongoing.

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Author contributions SZ, BL, and HW designed and wrote the paper. SZ, BL, HW, YZ, LS, and XZ performed the research.

Competing interests The authors declare that they have no competing interests.

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