In vitro and in vivo efficacies of artesunate in the treatment of cystic echinococcosis

LM Wen
Xinjiang Medical University Affiliated First Hospital
GD Lv
Xinjiang Medical University Affiliated First Hospital
J Zhao
Xinjiang Medical University Affiliated First Hospital
S Lu
Xinjiang Medical University Affiliated First Hospital
YH Gong
Xinjiang Medical University Affiliated First Hospital
YF Li
Xinjiang Medical University
HY Zheng
Xinjiang Medical University
B Chen
Xinjiang Medical University Affiliated First Hospital
HJ Gao
Xinjiang Medical University Affiliated First Hospital
CY Tian
Xinjiang Medical University
JH Wang (jhw716@sohu.com)
Xinjiang Medical University Affiliated First Hospital

Research article

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Abstract

Background: In this study, we aim to investigate the efficiency of artesunate (AS) in treating cystic echinococcosis (CE).

Methods: Eosin staining and transmission electron microscope (TEM) were utilized for the evaluation of treatment efficiency. DHR123 method and comet assay were utilized to detect reactive oxygen species (ROS) content and DNA damages.

Results: Both in vivo and in vitro experiments showed that AS showed significantly higher anti-parasitic effects on CE compared to albendazole (ABZ), especially under in vitro conditions. AS could elevate the ROS level in the protoscoleces (PSCs), which resulted in obvious DNA damages. AS could significant improve the liver function in infected mice compared with the model group ($P < 0.05$). Moderate and high doses of AS could decrease the TNF-α content compared with the model group ($P < 0.05$). The content of H2O2 in hydatid fluid treated by AS showed significant decrease compared with the model group ($P < 0.01$), while the T-SOD level showed significant elevation compared with model group ($P < 0.01$).

Conclusions: In this study, we confirmed that the effects of AS on PSCs may be related to the DNA damages induced by oxidative stress, which provided solid information for the research and development of drugs for hydatid disease.

Background

Cystic echinococcosis (CE) is an endemic helminthic disease induced by metacestodes (larval stage) infection of *Echinococcus granulosus* tapeworm **[1]**. The life cycle of CE involves definitive hosts (e.g. dogs) for the intestinal tapeworm, together with the domestic and wild ungulates as intermediate hosts for the tissue-invading metacestode **[2]**. CE infection is closely related to the direct hand-to-mouth transfer with the infected definitive hosts, egg-containing feces, or egg-contaminated plants or soil **[3]**. Nowadays, *E. granulosus* is still widely distributed, especially in the Mediterranean countries, Russia and western China **[4]**.

The main treatment options for CE include surgery and chemotherapy. To date, surgery is preferred for the treatment in clinical settings, however, a higher recurrence rate is reported as the pathological features of CE are rather complex **[4]**. To our best knowledge, surgery is only suitable for those with early-stage cases with a few hydatid cysts. Meanwhile, it is still a challenge in the remote areas due to limited medical resources. For

the majority of cases, chemotherapy agents are necessary to improve the life quality of patients with a tendency of recurrence, multiple-organ involvement as well as the cases of the advanced stages.

Currently, benzimidazoles (e.g. mebendazole and albendazole) are considered as the first option for chemotherapy, and are applied in high doses for extended periods of time. This frequently results in adverse events. The most adverse events of benzimidazole compound including gastro-intestinal disturbances such as leukopenia, liver injury after long-term use, embryo toxicity and malformation leukocyte reduction **[5]**. To date, albendazole (ABZ) is the only drug recommended by WHO for treating human CE **[6]**. Limited and low efficient chemotherapeutic options are stumbling blocks to the treatment of CE. Hence, it is urgent to develop novel and effective chemotherapeutic agents.

Artemisinin (ART), an anti-malarial agent discovered in the 1970s by Professor Youyou Tu, shows high efficacy and low toxicity for cancer patients **[7]**. The active moiety of ART is a sesquiterpene lactone containing an endoperoxide bridge that can lead to generation of reactive oxygen species (ROS) responsible for mediating cytotoxic action of artemisinin derivatives in the parasites **[8]**. According to the recent data, ROS was distributed in mitochondrial compartment in the parasites, which then resulted in impaired mitochondrial functions and DNA damages **[9, 10]**. The anti-CE activity of ART derivatives involves direct DNA damage and subsequent parasite death. As the semi-synthetic derivative of ART, Artesunate (AS) has been reported to involve in DNA damage and repair processes **[11]**. In *E. granulosus*, we identified the ribosomal protein S9 (RPS9) from *E. granulosus* (EgRPS9) involved in DNA repair pathways **[12]**. To date, many pharmacological actions and cellular targets have been identified **[13-15]**, but the anti-CE effects of AS are still ambiguous. In this study, we aim to investigate the pharmacodynamic roles of AS against CE and the potential mechanisms.

Methods

Isolation of parasites

1. *granulosus* protoscoleces (PSCs) were aseptically isolated from liver hydatid cysts obtained from infected sheep scheduled for routine slaughter in an abattoir located in Urumqi (Xinjiang Autonomous Region, China). PSCs were aspirated from liver hydatid cysts, followed by pepsin-activation and *in vitro* culture according to our previous description **[12]**.

Evaluation of pharmaceutical efficiency of AS on PSCs

After culturing for 24 hrs, the PSCs were collected and washed with PBS thrice. PSCs with a viability of ≥95% were used for the subsequent analysis. About 250 PSCs were seeded onto each well of the 96-well plates by dividing into the following groups: control group, plates supplemented with cultivation medium; DMSO group, treated with dimethyl sulfoxide (DMSO, 1%, v/v); H_2O_2 group, treated with 50 μM H_2O_2 (as a positive control for ROS); ABZ group (25 μM, as positive drugs); and AS groups, PSCs were treated with different concentrations of AS (65 μM, 130 μM, and 325 μM), respectively. After treating for 4 days, PSCs were collected to evaluate the mortality via the eosin dye exclusion test, followed by monitoring the ultramicroscopic change by transmission electron microscopy (TEM, JEM-100CXII, Alignment, Japan) as previously described **[16, 17]**.

Effect of AS on E. granulosus mortality in vitro

PSCs were divided into the following groups: DMSO group; H_2O_2 group; ABZ group (25 μM); and AS high dose group (325μM, AS-H); H_2O_2 plus Mannitol group, treated with 50 μM H_2O_2 and 100 μM mannitol (scavenger of ROS); AS-H plus Mannitol group, treated with 325 μM AS and 100 μM mannitol. After treating for 4 days, PSCs were collected to evaluate the mortality and observe the ultramicroscopic changes.

Detection of ROS content in PSCs of E. granulosus

Generation of ROS in PSCs was determined as previously described **[18]**. Briefly, the dihydrorhodamine 123 (DHR123, D1054, Sigma-Aldrich) was added to the 96-well plates covered with PSCs to a final concentration of 10 μM. Then the mixture was incubated at

37°C for 30 min. Upon removal of the supernatant, the mixture was washed using PBS once, followed by incubating with agents at 37°C. The wavelength was set at 530 nm (λex = 488 nm). A multifunction reader (Thermo Fisher) was utilized for the observation for 5.5 hrs every 30 min.

Detection of DNA damage by comet assay

Optimized comet assay for *E. granulosus* was carried out according to method for *Plasmodium falciparum* described by Gopalakrishnan *et al* **[19]**. The PSCs were treated for 24 hr, the culture medium was abandoned and washed using PBS. Normal-melting point agarose (NMA) was transferred onto the slide, and incubated overnight at 65°C to solidify the agarose. Diluted PSCs (100) were mixed with 100 µl low-melting point agarose (LMA) added onto the slides which were then immediately covered with coverslips. After agarose solidification at 4 °C for 5 min, the coverslips were removed and the slides were immersed for 4 hrs at 4 °C in freshly lysis solution. The slides were equilibrated in alkaline solution for 20 min. Electrophoresis was carried out for 20 min at 25 V and 300 mA. Afterwards, slides were neutralized by washing with Tris-HCl buffer for 15 min, and were stained with 60 µl propidium iodide (PI) solution in dark. Finally, the images were observed using a fluorescent microscope (Olympus IX73) connected through a CCD-IRIS Color Video Camera (Hitachi Denshi, Japan), under a magnification of 200×. The image for PSCs was acquired immediately after opening the microscope shutter to the computer monitor, employing the CASP Program.

Experimental infection and in vivo treatment

Female Kunming mice (6 weeks old) provided by the Animal Center, Xinjiang Medical University, were subject to adaptive feeding for one week before experiments. All protocols involving animals were approved by the Animal Welfare and Committee of First Affiliated Hospital of Xinjiang Medical University (IACUC-20150225-70). Mice were infected by intraperitoneal injection 25 small vesicles with a diameter of 250-300 µm cultured from PSCs of *E. granulosus in vitro* as previously described **[20, 21]**. Six months later, selection of infected mice was performed under ultrasonographic examinations.

The infected mice were randomly divided into: (i) model group (n=10): treated with Tween-80/0.4 % CMC-Na; (ii) ABZ positive control group (n=10): treated with ABZ (200 mg/kg); and (iii) AS groups (n=30): treated with 50 mg/kg($1/20LD_{50}$), 100 mg/kg ($1/10LD_{50}$) and 200 mg/kg ($1/5LD_{50}$) of AS. Uninfected mice treated with an equal volume of 0.4 % CMC-Na served as control group (n=10). The solution was given by intragastric administration lasting for 6 weeks.

Sample collection and detection

Six weeks later, the blood samples were obtained by cardiac puncture after anesthesia under anesthesia with pentobarbital sodium administered intraperitoneally [22]. Liver function was assessed by determining the content of total bilirubin (TBIL) and direct bilirubin (DBIL) levels, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using the commercial kits (Jiancheng Biotech, Nanjing, China), according the manufacturer's instructions. Moreover, serum TNF-α and GSH/GSSG was determined to evaluate the oxidative stress status. The mice were sacrificed by cervical dislocation immediately after blood collection, and then the cysts were isolated and subject to weighing the wet weight of cysts. The cystic fluid was obtained and then the content of superoxide dismutase (SOD) and H_2O_2 was determined. The cystic wall was observed using TME.

Statistical analysis

Data analysis was performed using SPSS 18.0, and measurement data were presented as mean ± standard error, and numeration data were presented as percentages. The comparison was analyzed with one-way ANOVA and χ^2 test, respectively. $P < 0.05$ was considered to be statistically significant.

Results

Pharmacodynamic features of AS on PSCs

Compared with the DMSO group significant increase was observed on day 4 in the mortality in the low (52.81 ± 3.34% vs. 13.92 ± 0.60 %, $P < 0.01$), moderate (70.06 ±

3.67% vs. 13.92 ± 0.60 %, $P < 0.05$) and high AS groups (76.84 ± 4.75 % vs. 13.92 ± 0.60 %, $P < 0.05$), respectively (Figure 1A). It presented a dose-dependent manner. Compared with the ABZ group, significant increase was noticed in the mortality was observed in the low, moderate and high dose AS groups ($P < 0.05$). On day 4, in the AS groups, the laminar layer (*ll*) were intact, together with the microtrichies (mt). There were no aberrant changes in the parenchyma cells, and the structures were clear (Figure 1B). In the ABZ group, the mt was no longer available, the membrane structure was turbid. The cell nucleus was no longer available. Lipid droplets were observed. In the low AS group (AS-L group), few microvillus were observed, and the nucleolus was disperse. In the AS-M group, there were some heterochromatins. In the AS-H group, there were massive heterochromatins. This indicated that AS could interrupt the karyotin in the PSCs.

Effects of AS on the DNA oxidative damages in PSCs

The mortality in the DMSO group and ABZ group was 13.9±0.60 % and 31.55 ± 0.95 %, respectively (Figure 2A). Compared with the H_2O_2 group, significant decrease was observed in the H_2O_2+Mannitol group ($P < 0.01$). Compared with the AS-H group, the mortality in the AS-H plus Mannitol group showed significant decrease ($P <0.01$). For the ultrastructure, Mannitol could significantly attenuate the injuries of mt, laminar layer and nucleolus by H_2O_2+AS (Figure 2B). In Figure 2C, we determined the content of ROS within 30 min after AS treatment, which indicated that AS could trigger the elevation of ROS in PSCs. Significant decrease was observed in the ROS content in the AS combined with mannitol group ($P < 0.01$). Comet assay revealed that AS could induce obvious DNA damages in PSCs (Figure 2D). After combing with the mannitol, obvious decrease was observed in the DNA injury damages ($P < 0.01$). These implied that mannitol could inhibit the anti-parasitic effects of AS on PSCs, which indicated that the anti-parasitic effects of AS may be related to the ROS-associated DNA injuries.

Pharmacodynamic features of AS against CE

Upon drug treatment for 6 weeks for the CE infected mice, the animals were sacrificed by CO_2 euthanasia, and cyst tissue removed from the peritoneal cavity. Cyst weight in the model group was 6.76 ± 2.51g, while that of the ABZ group was 1.76 ± 0.97g. The cyst weights in the AS-L, AS-M and AS-H groups were 4.3 ± 1.26g, 3.29 ± 1.4g, and 2.99 ± 2.19, respectively. Significant improvements were obtained after treating with ABZ, AS-L, AS-M and AS-H as revealed by one-way analysis of variance; $P < 0.01$). In addition, upon obtaining the hydatid fluid, the cystic wall was observed under the TEM for monitoring the ultrastructure (Figure 3). The germinal layer in the model group was clear. The mt was intact and massive in number. The nucleus was large and round. The nucleus was clearly displayed. The laminar layer structure was even. The mt in the ABZ group and AS-L group was clear. The laminar layer, germinal layer, layer structure and cellular structure were clear. The layer structure in the AS-M group showed swelling, together with lipid droplet. The structure of the AS-H group was not regular, and the mt was no longer available. Massive lipid droplets were observed. The severity of germinal layer and layer structure increased after AS treatment, which was presented in a dose-dependent manner. The injury of the cystic wall tissues in the AS-H group was higher than that of the ABZ group.

Effects of AS on the liver function and oxidative damage in CE

Compared with the model group, the TBIL and AST levels showed significant decrease in the AS groups ($P < 0.05$). Compared with the model group, significant decrease was noticed in the DBIL and ALT in the AS-M and AS-H groups ($P < 0.05$). Compared with the ABZ group, significant decline was observed in the TBIL in the AS-M and AS-H groups ($P < 0.01$). There were no statistical differences in the DBIL, ALT and AST among the AS groups ($P > 0.05$). In addition, ABZ and AS could improve the liver function. The liver protective effects of AS against mice with CE were similar with the ABZ.

The serum TNF-α showed elevation in mice infected with hydatid cysts (Figure 4). Compared with the model group, the TNF-α content in the AS-L groups declined with statistical differences were noticed ($P < 0.05$). In contrast, significant decrease were noticed in the TNF-α in the AS-M group compared with the model group ($P < 0.01$). The

TNF-α in the AS-H group showed significant decline compared with the model group ($P < 0.01$). The TNF-α showed significant decline in the AS-H group compared to that of the ABZ group ($P < 0.01$). Serum GSH/GSSG assay revealed that the oxidative stress showed a tendency of decrease in the infected mice treated with AS (Figure 4). Compared with the model group, there were significant decrease in the serum oxidative stress in the AS-H group ($P < 0.05$). AS could decrease the content of H_2O_2, compared with the model group ($P < 0.01$). Compared with ABZ group, AS could significant decrease the content of H_2O_2 ($P < 0.01$). In the mice infected with CE, the T-SOD content in the cystic fluid showed increase to some extent. Compared with the model group, T-SOD showed significant increase in the ABZ group and AS groups ($P < 0.01$). There were no statistical differences in the T-SOD between the AS group and ABZ group.

Discussion

To our knowledge, we evaluated the effects of AS *in vitro* by eosin staining, and *in vivo* by wet weight of cysts from mice. We also investigated the ROS generation by DHR123 method, DNA damage by comet assay, respectively. In addition, mannitol was used as ROS scavenger **[8]** to identify the potential mechanisms of how ART to play an anti-echinococcosis role. Our data showed that AS showed anti-echinococcosis effects in *vitro* and *in vivo*. Meanwhile, ROS level showed elevation after AS treatment, which presented in a dose-dependent manner. Similarly, comet assay showed that H_2O_2 and AS could induce DNA damages in PSCs. The DNA damage showed attenuation in the combination of AS combined with ROS scavenger, and the mortality showed significant decline. These data implied that the anti-parasitic effects of AS on PSCs may be related to the DNA damages induced by ROS generation. This paves the way for the development of new drugs for the screening of targets for the hydatid cysts based on AS.

Comet assay, initially reported by Ostling *et al* in 1984, is utilized in the determination of DNA strand damages **[23]**. However, it is not used in the evaluation of DNA damages in this species of *Echinococcus granulosus*. In this study, it is used for the evaluation of PSCs for the first time after multiple optimizations, including the gel procedures, time for the lysis,

staining dye selection, as well as the rinse frequency and time. This paves the way for the comet assay for the DNA damage evaluation in the PSCs.

In this study, wet weight of cysts and ultrastructure evaluation under TEM were used as the indices for evaluating the *in vivo* potency. Our data showed that AS could inhibit the lesion growth of the CE in mice. Besides, the studies on CE are hampered by the parasitism of cysts in the hosts. In this study, cystic fluid was preferred for the determination of indices associated with oxidative stress, in order to investigate the potency of AS. Preliminary tests showed that only T-SOD and H_2O_2 were measurable, and only the H_2O_2 showed decrease in the fluid in the AS groups compared with the ABZ group. Whereas, the T-SOD activity in AS groups was significantly higher than that of ABZ group, indicating that AS could promote the anti-oxidant capacity of PSCs. On this basis, we speculated that AS induced remission of hydatid disease may be related to the oxidative stress improvement in the cysts.

Indeed, there are some limitations in this study. For example, only T-SOD and H_2O_2 could be detected in the cystic fluid. As there were no PSCs in the EC in infected mice, we can not confirm the definite presence of DNA damages. We tried to verify our hypothesis through determining the 8-OHdG as previously described **[24]** in the cystic wall, however, there were no statistical differences. In our subsequent study, we will focus on the DNA damages of PSCs in the mice infected with CE, together with the determination of oxidative damage indices.

Conclusions

In summary, AS was superior to the ABZ in treating PSCs infection *in vitro*. AS could significantly increase the oxidative stress and DNA damages. In addition, the combination of AS and mannitol could significantly attenuate the DNA injuries, which implied that the potency of AS may be related to the regulation of DNA damages induced by oxidative stress in PSCs. The anti-CE effect of AS was not superior to ABZ *in vivo*. We confirmed that the effects of AS on PSCs may be related to the DNA damages induced by oxidative stress,

which provides solid information for the research and development of drugs for hydatid disease.

Abbreviations

AS: artesunate; CE: cystic echinococcosis; TEM: transmission electron microscope; ROS: reactive oxygen species; ABZ: albendazole; PSCs: protoscoleces; ART: Artemisinin; RPS9: ribosomal protein S9; NMA: Normal-melting point agarose; LMA: low-melting point agarose; PI: propidium iodide; TBIL: total bilirubin; DBIL: direct bilirubin, ALT: alanine aminotransferase; AST: and aspartate aminotransferase; SOD: superoxide dismutase

Declarations

Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WLM wrote the manuscript; WJH revised the manuscript; LGD, ZJ, LS, GYH did the data analysis; LYF, ZHY, CB, GHJ, TCY did the data collection. All authors have read and approved the manuscript.

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Figures

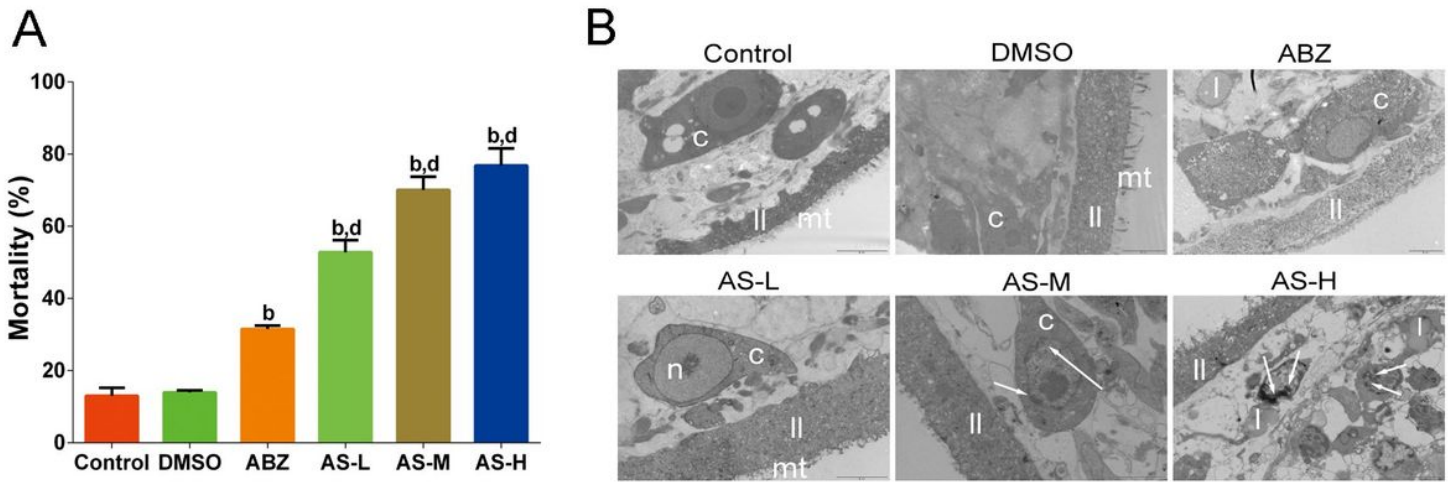

Figure 1

Effects of AS on mortality and ultrastructural changes of E. granulosus. (A) Mortality of PSCs on day 4 in each group using eosin dye exclusion test. (B) Ultrastructural changes by TEM. b P<0.01 versus DMSO group; d P<0.01 versus ABZ group

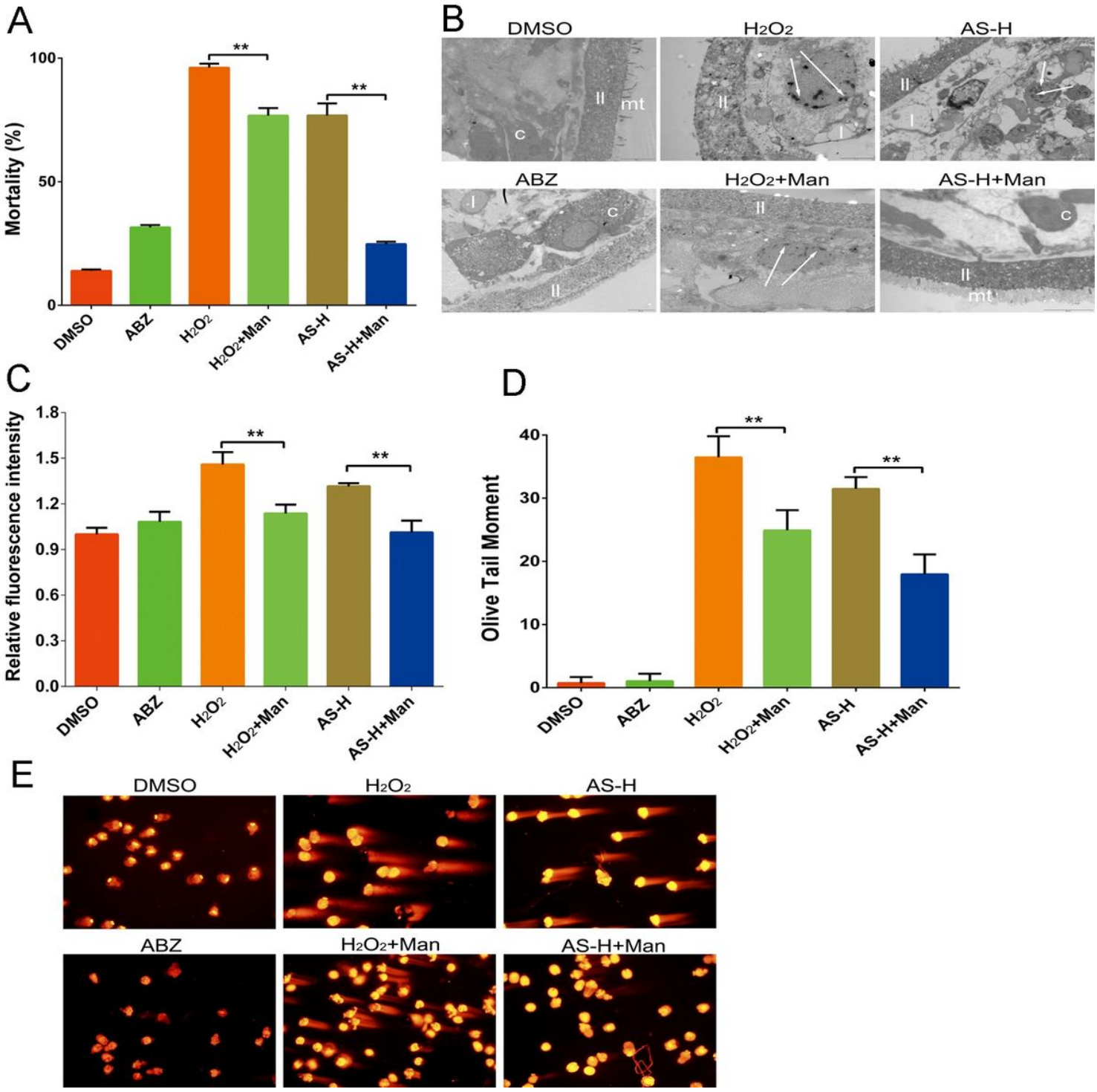

Figure 2

DNA oxidative damage effects of AS on E. granulosus. PSCs were treated 4 days and then collected to detect the mortality (A), and observe ultrastructural changes by TEM (B). ROS content in PSCs (C) tabbed by dihydrorhodamine 123, and DNA damage (D and E) established by Comet assay. **P < 0.01

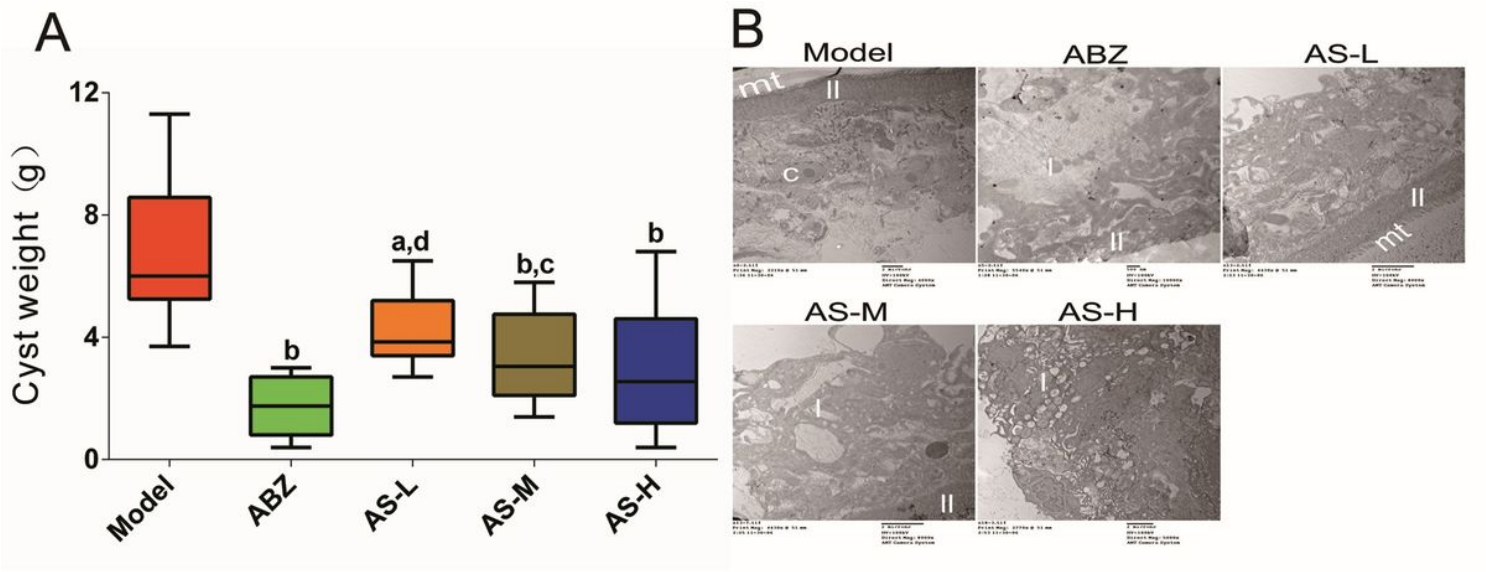

Figure 3

Experimental chemotherapy with E. granulosus-infected mice. (A) Cyst weight from mice at week 6. (B) Cyst wall observed under transmission electron microscope in model Group (4000×), ABZ group (5000×), AS-L (5000×), AS-M (5000×), and AS-H (8000×). a $P<0.05$ versus model group; b $P<0.01$ versus model group; c $P<0.05$ versus ABZ group; d $P<0.01$ versus ABZ group

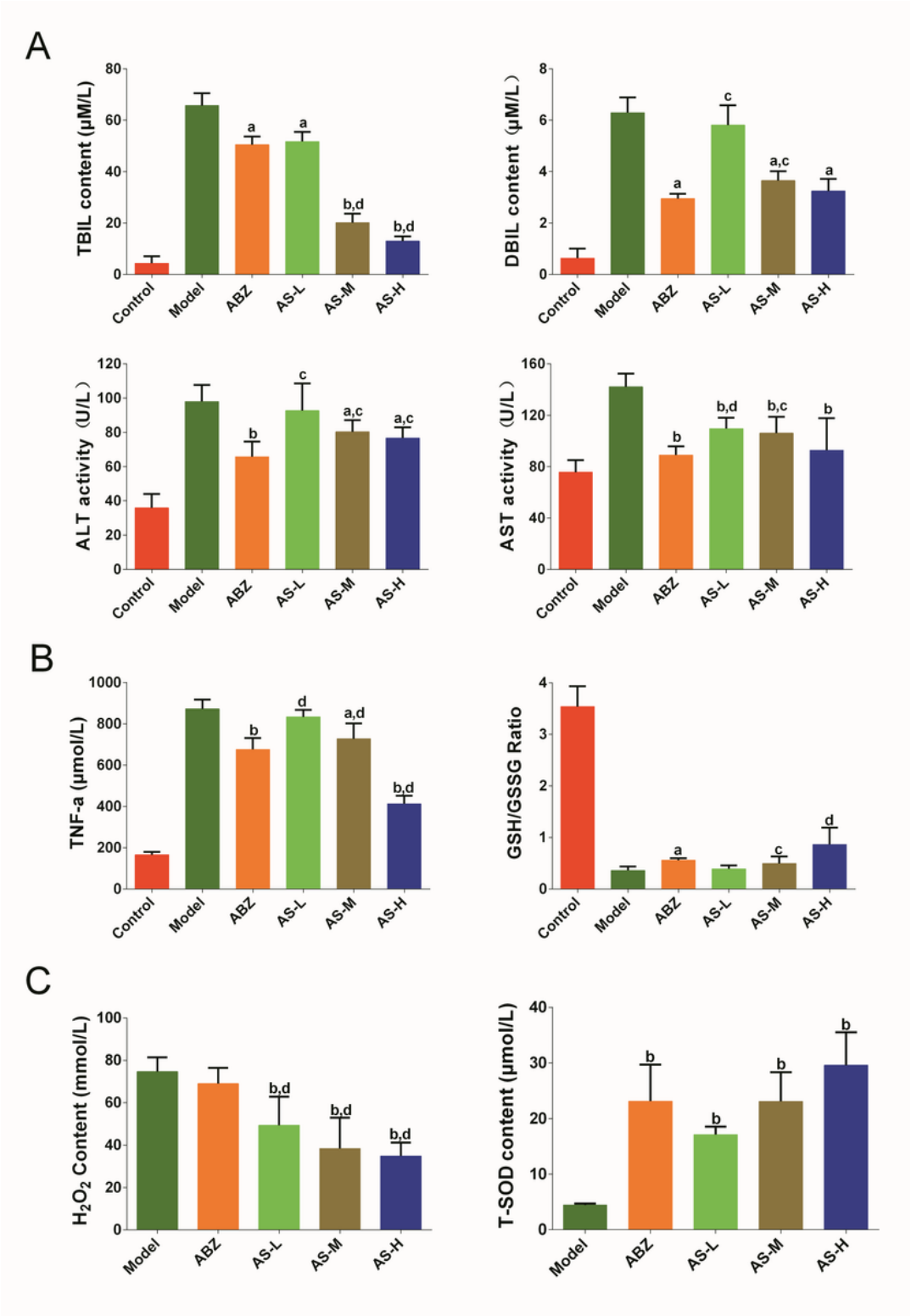

Figure 4

Molecular indices in serum or cyst fluid detection. (A) Liver function evaluation. (B) Molecular indices in serum detection. (C) Oxidative stress in cyst evaluation of mice in each experimental group. a P<0.05 versus model group; b P<0.01 versus model group; c P<0.05 versus ABZ group; d P<0.01 versus ABZ group

Supplementary Files

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