Expression and role of autophagy-related protein in the mice liver model of persistently infected with *Echinococcus multilocularis*

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Research Article

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Abstract

Objective The purpose of our study is to understand the changes of autophagy in the process of *Echinococcus multilocularis* infection through the detection of autophagy-related proteins Beclin1, LC3, and p62 in the liver of mice infected with *Echinococcus multilocularis.*

Methods Female BALB / c mice were randomly divided into experimental and control groups. The experimental group established a mouse model of *Echinococcus multilocularis* infection. Six mice were sacrificed at 8d, 30d, 60d, 90d, and 180d respectively. The pathological changes of mice liver were observed by HE staining, and the expressions of autophagy-related proteins Beclin1, LC3, and P62 in mice liver infected with *Echinococcus multilocularis* were detected by qRT-PCR, immunohistochemistry, and Western Blot respectively.

Results The expression of Beclin1, LC3, and p62 showed significant difference between experimental groups and the control group ($P<0.05$). From 8 days to 60 days after infection, the expression levels of Beclin1, LC3, and p62 in the liver of mice were gradually increased. At 90 days after infection, Beclin1 and p62 expression decreased, while LC3 still showed an upward trend. However, the expression of Beclin1, LC3, and p62 protein decreased significantly after 180 days of infection.

Conclusions Beclin1, LC3, and P62 in the liver of mice infected with *Echinococcus multilocularis* showed a trend of first increasing and then decreasing, and autophagy increased and then decreased with the infection.

1. Background

Hydatid disease is a zoonotic disease with serious harm. *Echinococcus multilocularis*, which can cause alveolar hydatid disease. The role of rodents as intermediate hosts is an important part of their life history. After the body is infected with echinococcus, the primary lesion is more common in the liver, showing invasive growth, and even spread to the abdominal cavity, so it is also called "worm cancer".[1] Autophagy is ubiquitous in eukaryotic cells, which is very important for cells to maintain their normal physiological process and is one of the important protection and defense mechanisms of the body. Changes in autophagy are closely related to diseases. Autophagy is the most primitive form of innate immunity for eukaryotic cells to remove invading pathogenic microorganisms.[2] Autophagy is an evolutionally-conserved process regulated by different autophagy-related genes.[3] Beclin1 is necessary for mammals to participate in the formation of autophagosomes during autophagy. LC3 expression level is related to autophagy activity and can be used as one of the specific diagnostic indicators of autophagy.[4] As an autophagy receptor, P62 is related to autophagy flux.[5] In this study, the expression changes of autophagy-related proteins Beclin1, LC3, and P62 in the liver of mice infected with echinococcosis were detected to explore the relationship between autophagy and persistent echinococcosis, to provide a new idea for the basic research and clinical treatment of echinococcosis.

2. Material And Methods

2.1 Material

2.1.1. Experimental animals

SPF grade BALB / c mice, female, 60, 6–8 weeks old, 18~22 g, provided by the Experimental Animal Science Research Department in First Affiliated Hospital of Xinjiang Medical University. All experimental procedures were conducted with strict guidelines and approval of Institutional Animal Use and Care Committee at Xinjiang Medical University (Approving Number: ZACUS-201304255002).

2.1.2. The main reagents and instruments

Beclin1 antibody, Goat Anti-rabbit IgG/HRP antibody was purchased from Bioss. LC3A/B Antibody,p62 Antibody was purchased from Affinity Biosciences. PrimeScript™ RT Master Mix (Perfect Real Time), TB Green® Premix Ex Taq™ II(Tli RNaseH Plus) was purchased from TAKARA. BCA Protein Assay Kit, SDS-PAGE Gel Kit was purchased from Solarbio.

2.2. Methods

2.2.1. Animals model establishment and grouping

BALB/ C mice infected with *Echinococcus multilocularis* were sacrificed under aseptic conditions. The *Echinococcus multilocularis* tissues in the liver were removed, then the necrotic tissues were separated. Aspirated liquid with 5 ml sterile syringe into 50 ml sterile centrifuge tube, residualed shear grinding, filtered with double-layer sterile gauze, washed several times in sterile normal saline containing 500 U/ml penicillin and 100 U/ml streptomycin until larvae deposit and lotion were clear. Then using phosphate-buffered saline (PBS) processed 10000 original larvae /ml for back-up. Mice were randomly divided into experimental group (n=30) and control group (n=30), each of experimental groups was received an intraperitoneal injection with 0.5ml original larvae liquid, and each of the control group was injected with normal saline of the same volume.

2.2.2 Specimen collection

At 8, 30, 60, 90, and 180 days after infection, mice in experimental group and control group were sacrificed by cervical dislocation. In the aseptic environment, the abdominal cavity was opened along the linea alba, and liver tissue was taken. Part of liver tissue was fixed in 4% paraformaldehyde solution for 72 hours, which was prepared for HE staining and immunohistochemical. The other part was put into a liquid nitrogen cryopreservation tube and transferred to - 80 ℃ refrigerator for extracting total RNA and protein.

2.2.3 HE Staining

Trim the fixed tissue, dehydrate in graded alcohols, and wash with dimethyl benzene, after routine paraffin embedding, the samples were continuously sliced at 4 μm on the polylysine pre-managed slides. For each sample, three slices were randomly selected for routine hematoxylin-eosin (HE) staining. Then the result was observed by microscope and the pictures were collected.

2.2.4 Immunohistochemistry

The slides were cleared by xylene conventionally, and dewaxed to water, placed in citrate buffer at 95 °C for 15 min, cooled to room temperature. then incubated with 3% hydrogen peroxide for 10 min, and added with goat serum working solution at room temperature for 30 min. Subsequently, primary antibodies against Beclin1 (1:50 dilution), LC3 (1:100 dilution), and p62 (1:100 dilution) were added respectively, incubated at 4 °C overnight. Placed for 1 hour at room temperature, incubated with Goat Anti-rabbit IgG/HRP antibody for 2 hours at room temperature, followed by coloring with the developer of 3, 3’-diaminobenzidine (DAB) for 1 min. and the plate was dehydrated quickly after re-staining with hematoxylin. Observed and taken pictures under the microscope. Stained with brown was positive, and the percentage of positive expression area was analyzed by Image J software.

2.2.5 qRT-PCR

The liver tissues in experimental group and control group were extracted about 20 mg each, and the total RNA was extracted by the Trizol method. Determine the OD260/280 value and record the concentration (the OD260/280 value was between 1.9 and 2.0). After adjusting the total liver RNA concentration to 1000 ng/μL, 1 μL was taken into PrimeScript™ RT Master Mix (Perfect Real Time) for reverse transcription to cDNA. Then added 2μL cDNA into TB Green® Premix Ex Taq™ II(Tli RNaseH Plus) with PCR Forward Primer and Reverse Primer, and RNase-free water. Hereafter, Beclin1, LC3, p62 mRNA expression were detected by 7500 Software (Applied Biosystems), and GAPDH was chosen as the internal reference. After the reaction, record the CT value of each group, and the target gene was quantified by $2^{-\Delta\Delta CT}$ relative quantitative method. The primers were synthesized by Sangon Biotech. The sequence of each primer is shown in Table. 1.

Table. 1 The sequence of autophagy-related protein forward primer and reverse primer

	Forward Primer sequence	Reverse Primer sequence
GAPDH	5'- TGGTGAAGCAGGCATCTGAG -3'	5'- TGAAGTCGCAGGAGACAACC -3'
Beclin1	5'- AGCCTCTGAAACTGGACACG -3'	5'- TTCCTCCTGGGTCTCTCCTG -3'
LC3A	5'- AGCGCTACAAGGGTGAGAAG -3'	5'- TCTTGGGAGGCGTAGACCAT-3'
LC3B	5'- AGATCCCAGTGATTATAGAGCGA -3'	5'- TTGCTGTCCCGAATGTCTCC -3'
p62	5'- ACAGCCAGAGGAACAGATGG -3'	5'- ACTGGAGTTCACCTGTAGATGG -3'

2.2.6 Western Blot Analysis

About 20 mg liver tissues was taken out both in experimental group and control group, and put into the glass homogenizer, added with 300 μL RIPA buffer and 3 μL phenylmethylsulfonyl fluoride (PMSF). After

sufficient homogenization, the samples were stood on ice for 30 min, then centrifuged for 15 min by the high-speed refrigerated centrifuge under 12000 r/min. The protein concentration in the supernatant was determined by BCA Protein Assay Kit and diluted to 2 μg/μl with 4x protein loading buffer and PBS, and then boiled at 100℃ for 10 min. Equal amounts of the proteins were subjected to SDS-PAGE (12%) under reducing conditions; the separated proteins were transferred to PVDF membranes, then blocked with 5% skim milk for 2 h, the membranes were incubated with antibodies against Beclin1 (1:500 dilution), LC3 (1:500 dilution), and p62 (1:1000 dilution) overnight at 4℃. The secondary antibody (Goat Anti-rabbit IgG/HRP antibody) were incubated at room temperature for 1.5 h. After reaction with ECL luminescent for 1 min, the membranes were detected the signals by the chemiluminescence detection system and analyzed by image lab software (American Bio-Rad company). The gray values of protein bands were calculated by normalization to GAPDH which acts as the internal reference.

2.2.7Statistical Analysis

All data are presented as mean ± Standard Deviation. Differences among groups were compared using one-way ANOVA. Statistical analyses were performed by using SPSS 22 and statistical significance was set at $P<0.05$. mapping was carried out by GraphPad Prism 8 software.

3. Results

3.1 Anatomical observation of mice infected with *Echinococcus multilocularis* at different stages and vesicle growth score

No abnormal changes in the liver and abdominal cavity were observed after dissection in control group mice at different periods. The liver was soft, smooth, red in color, no obvious adhesion, and the anatomical position of the liver was normal. (Fig. 1-F)In experimental group, after 8 days of infection with *Echinococcus multilocularis*, there was no significant difference between experimental group and control group. (Fig. 1-A) After 30 days of infection, it could be found that the liver surface was smooth, with slightly dull color and needle-tip pinpricks. (Fig. 1-B) After 60 days of infection, small vesicles can be observed on the surface of the liver, and the liver was slightly larger and dimmer in color. (Fig. 1-C) At 90 days after infection, many vesicles were diffused in the abdominal cavity of mice, varying in size, mostly formed by the fusion of small vesicles. The liver was slightly hard in texture, enlarged in volume, and dim in color. There was partial adhesion with surrounding tissues and abdominal cavity, and the anatomical position had deviated from the normal position. (Fig. 1-D) At 180 days of infection, there were a large number of vesicles in the abdominal cavity of mice, necrosis, and calcification in the vesicles, and the boundary with eroded tissues was blurred. The liver eroded by vesicles is dim in color, hard in texture, obviously adhering to the surrounding organs and not easy to separate, and has deviated from the normal anatomical position obviously. (Fig. 1-E) vesicle growth score was showed in Table. 2, Fig. 2.

Table. 2 Vesicle growth score in mice infected with *Echinococcus multilocularis*

Average mass of vesicles（g）	Occupying the number of hepatic lobes（）	Involving other organs	score
10	6		4
5-10	4-5	YES	3
2-5	2-3		2
2	1		1
0	0	NO	0

3.2 Pathological changes and pathological scores of the liver in mice infected with *Echinococcus multilocularis* at different stages

In the control group, the structure of hepatic lobules was intact, the boundary of hepatocytes was clear and arranged neatly. Occasionally, the cytoplasm of hepatocytes became loose and inflammatory cells accumulated. (Fig. 3-F) In the experimental group, at 8 days after infection, the structure of hepatic lobules was intact, hepatocytes appeared edema, and inflammatory cell infiltration began to appear in the portal area. (Fig. 3-A) At 30 days after infection, the structure of hepatic lobules was intact, spotty necrosis of hepatocytes could be seen, and inflammatory cell aggregation appeared around the portal area obviously. (Fig. 3-B) At 60 days after infection, inflammatory cell infiltration continued to aggravate in the portal area and vesicle wall formation could be seen. (Fig. 3-C) At 90 days after infection, inflammatory cell aggregation and bridging necrosis around the lesion could be seen, and the structure of hepatic lobules was disordered. (Fig. 3-D) At 180 days after infection, the normal hepatic lobular structure was no longer visible, and a large number of inflammatory cells infiltrated around the lesion. (Fig. 3-E) Histopathological Score was showed in Table. 3, Fig. 4.

Table. 3 Histopathological Score of Liver in Mice Infected with *Echinococcus multilocularis*

Degree of inflammation		Abnormal structures and substances	score
Hepatic lobule	Port area and surrounding		
Widespread bridging necrosis involving multiple lobules	Severe (inflammatory cells occupy >2/3 portal area)	brood capsule and protoscolex	4
Degeneration, fusion necrosis or bridging necrosis	Moderate (inflammatory cells occupy 1/3 to 2/3 portal area)	cyst wall(cuticular plate and germinal layer)	3
Degeneration, most spotty necrosis and focal necrosis			2
Degeneration, a few spotty necrosis or focal necrosis	Mild (inflammatory cells occupy <1/3 portal area)	granuloma	1
no inflammation present	no inflammation present	no obvious abnormal structure or substance	0

3.3 Distribution and positive expression rate of autophagy-related proteins

As shown in immunohistochemistry, the expression of Beclin1, LC3, and p62 protein in the liver of control group was little or no expression. The scattered positive expression could be observed at 8 and 30 days

after infection. at 60 and 30 days after infection, dense positive expression was observed at the junction of the focus and hepatocytes. (Fig. 5, Fig. 6, Fig. 7) Positive expression percentage of Beclin1, LC3, and p62 showed significant differences between the control group and experimental groups (P<0.05). (Table.4)

Table. 4 Positive expression percentage of autophagy related proteins in mice liver at different stages of infection（x±s,n=6) [a] Significant difference (P<0.05) between the experimental groups and the control group.

Days after infection	Beclin1	LC3	P62
8d	0.34±0.07[a]	0.24±0.06 [a]	0.24±0.07 [a]
30d	1.62±0.17 [a]	0.53±0.09 [a]	0.5±0.08 [a]
60d	2.22±0.14 [a]	1.78±0.21 [a]	2.27±0.21 [a]
90d	1.64±0.13 [a]	2.1±0.3 [a]	1.82±0.2 [a]
180d	0.42±0.13 [a]	0.3±0.08 [a]	0.2±0.04 [a]
control group	0.11±0.01	0.1±0.02	0.1±0.01

3.4 mRNA expression of autophagy-related protein mRNA at different stages of infection

In qRT-PCR, the expression of Beclin1 showed a significant difference at 30, 60, 90, 180 days after infection, compared with the control group (P<0.05). As for LC3, showed a significant difference at 30, 60, 90 days after infection compared with the control group (P<0.05). As for p62, showed a significant difference at 30, 60, 90, 180 days after infection, compared with the control group (P<0.05). (Table. 5, Fig. 8)

Table. 5 mRNA expression of autophagy-related proteins in mice liver at different stages of infection （x±s,n=6) [a] Significant difference (P<0.05) between the experimental groups and the control group.

Days after infection	Beclin1	LC3B/A	P62
8d	1.13±0.19	0.79±0.22	1.22±0.16
30d	1.46±0.26[a]	1.88±0.17 [a]	1.6±0.12 [a]
60d	1.56±0.14 [a]	3.1±0.96 [a]	2.27±0.47 [a]
90d	1.36±0.1 [a]	3.48±1.27 [a]	1.59±0.09 [a]
180d	0.5±0.09 [a]	0.69±0.17	0.57±0.11 [a]
control group	0.99±0.13	0.98±0.21	0.97±0.07

3.5 Expression of autophagy-related protein at different stages of infection

As shown in western blot , the expression of Beclin1 showed a significant difference at 30, 60, 90, 180 days after infection, compared with the control group (P<0.05). As for LC3A, showed a significant difference at 8, 30, 60, 90, 180 days after infection compared with the control group (P<0.05). As for LC3B, showed a significant difference at 30, 60, 90 days after infection compared with the control group (P<0.05). As for p62, showed a significant difference at 30, 60, 90, 180 days after infection, compared with the control group (P<0.05). (Table.6, Fig. 9)

Table. 6 Expression level of autophagy-related proteins in mice liver at different stages of infection (x±s,n=6) [a] Significant difference (P<0.05) between the experimental groups and the control group.

Days after infection	Beclin1	LC3A	LC3B	P62
8d	0.59±0.015 [a]	0.26±0.012 [a]	0.07±0.003	0.13±0.015 [a]
30d	0.77±0.011 [a]	0.47±0.02 [a]	0.12±0.029 [a]	0.12±0.023 [a]
60d	0.96±0.017 [a]	0.47±0.021 [a]	0.25±0.002 [a]	0.44±0.024 [a]
90d	0.9±0.018 [a]	0.61±0.017 [a]	0.39±0.002 [a]	0.35±0.026 [a]
180d	0.75±0.018 [a]	0.18±0.022 [a]	0.07±0.002	0.23±0.024 [a]
control group	0.71±0.007	0.13±0.014	0.06±0.002	0.08±0.01

4. Discussion

Parasites are important pathogens except for bacteria and viruses. They stimulate host cells and maintain a balance between inducing and evading host immune response, and host cells also maintain a balance between infection and elimination. Autophagy is a kind of innate immune system in cells. [6]When pathogens invade the body, the most direct way to eliminate pathogens by autophagy is to be engulfed by autophagy bodies, and then kill pathogens by lysosome autophagosome fusion.[3] Beclin1 gene is a homolog of yeast atg6, a mammalian specific gene involved in autophagy, and the central component of the phosphatidylinositol-3-kinase (PI3K) complex that initiates autophagy.[7]

LC3 is a homologous gene of Atg8 in mammals. LC3 is widely used as a marker for autophagic body detection. LC3 can be divided into PE bound LC3 (LC3B) and unbound LC3 (lc3a). LC3B content is also widely used to quantify autophagy activity.[3] However, LC3B expression does not reflect the downstream process of autophagy. Another autophagy-related protein p62 is involved in this process. As an autophagy receptor, p62 will be degraded continuously by autophagy in normal autophagy. The increase of LC3B expression is accompanied by a decrease of p62. Under the condition of autophagy substrate accumulation, the transcription of p62 is also significantly increased.[5] Therefore, it is necessary to detect Beclin1,LC3B and p62 simultaneously.

Researches in the field of other parasites, when Van Grol studied the mechanism of host cell CD40 participating in anti-*Toxoplasma gondii* immunity, he found that the anti-insect immunity of CD40 induced the occurrence of autophagy, and CD40 stimulated the production of autophagy-related proteins Beclin1 and LC3B, which enhanced autophagy, and killed *Toxoplasma gondii* with the cooperation of Atg7.[8] When using human umbilical cord mesenchymal stem cells (HUC MSc) as a model of congenital diseases, Chu et al. found that the invasion of *Toxoplasma gondii* to HUC MSc increased in a time-dependent manner with the increase of time, LC3B gradually increased with the extension of infection time, and p62 was down.[9]

And in other liver diseases, Zheng et al. found that the expression of autophagy-related proteins Beclin1 and *LC3B* increased gradually from hepatitis to chronic acute liver failure. With the development of the disease, hepatitis B virus replication increased. The higher the viral load, the higher the expression level of autophagy proteins Beclin1 and LC3B.[10] Yu and others studied the expression of autophagy-related proteins in hepatocellular carcinoma, they found that the positive expression rates of Beclin1 and LC3 proteins in 56 liver cancer tissues and 40 normal liver tissues were significantly higher than those in normal liver tissues, and the expression of Beclin1 and LC3 was positively correlated in liver cancer.[11]

At present, few studies have investigated the expression and role of Beclin1, LC3 and p62 in the process of Echinococcus infection. In this study, it was found that in mice infected with *Echinococcus multilocularis* from 8 days to 60 days, the expression levels of Beclin1, LC3, and p62 in the liver of mice were gradually increased with the deepening of infection, indicating that the formation of autophagy bodies and autophagy activation were increasing, which may be related to the fact that autophagy inhibits or eliminates pathogens in the early stage of pathogen infection and protects the body from the invasion of pathogens. At 90 days after infection, Beclin1 and p62 expression decreased, while LC3 still showed an upward trend, indicating that autophagy flux was increasing at this time. However, the expression of Beclin1, LC3, and p62 protein decreased significantly after 180 days of infection, and the PCR results were even lower than that in the normal control group. At this time, both autophagy activation and autophagy flux were significantly reduced, which may be related to immune escape, inhibition or blocking of autophagy. However, more studies are needed to explore the relationship between autophagy and persistent infection of *Echinococcus multilocularis*.

5. Conclusions

The expression of Beclin1, LC3, and p62 showed significant difference between experimental groups and the control group. Beclin1, LC3, and P62 in the liver of mice infected with *Echinococcus multilocularis* showed a trend of first increasing and then decreasing, and autophagy increased and then decreased with the infection.

Declarations

Ethics approval and consent to participate

SPF grade BALB / c mice provided by the Experimental Animal Science Research Department in First Affiliated Hospital of Xinjiang Medical University. All experimental procedures were conducted with strict guidelines and approval of Institutional Animal Use and Care Committee at Xinjiang Medical University (Approving Number: ZACUS-201304255002).

Consent for publication

Not applicable

Availability of data and materials

Data is available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Cai conceived of the study, participanted in the study design and drafted the manuscript. Aibibula participanted in the study design, carried out the experiments and drafted the manuscript. Setewaledi and Li helped to perform the experiments . ZHAO, Liu, Shan, and Qi helped to modify the manuscript; Cao and Ma conceived of the study and modify the manuscript. All authors read and approved the final manuscript.

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Figures

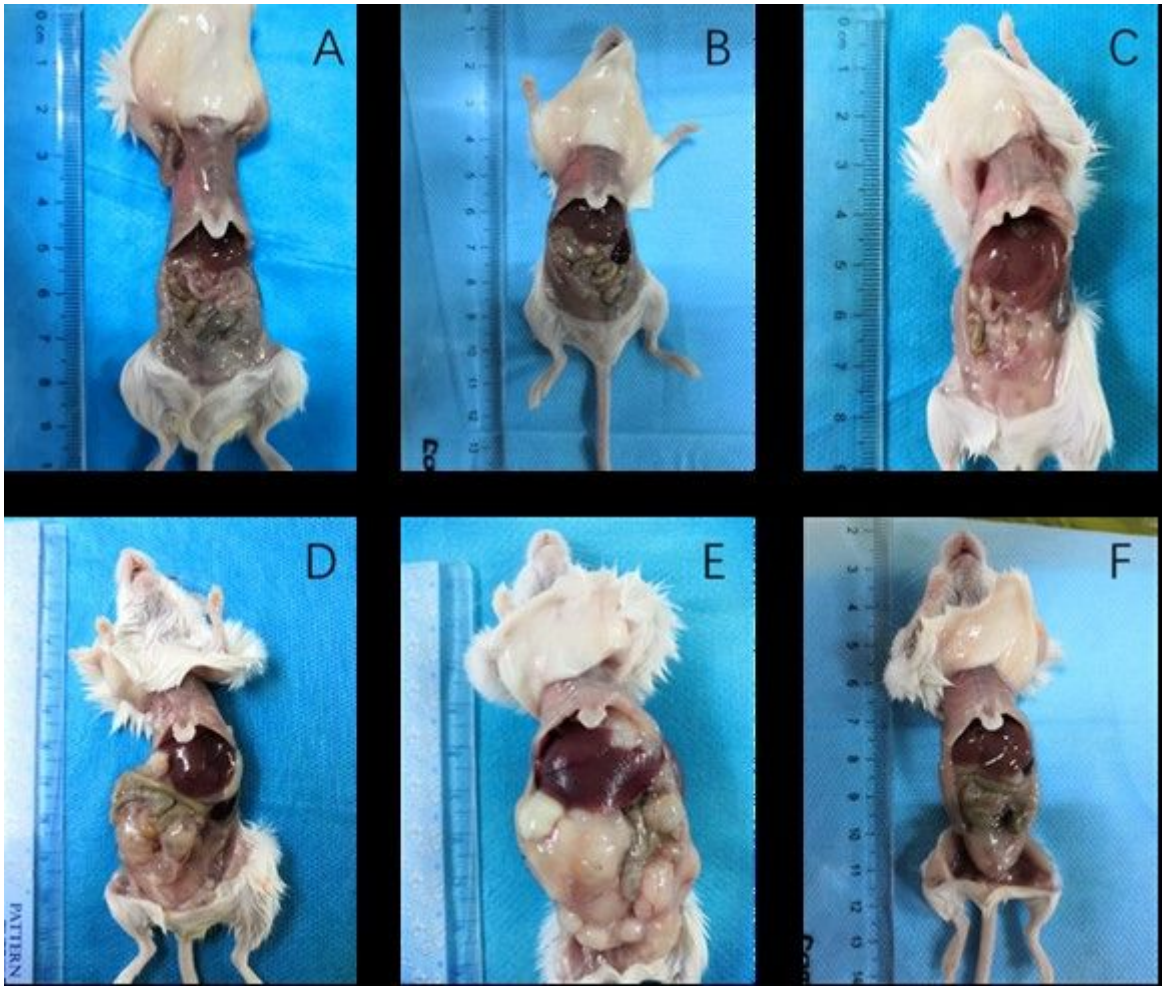

Figure 1

Anatomy of Mice at Different Stages of Infection with Echinococcus Figure A: At 8 days after infection. Figure B: At 30 days after infection. Figure C: At 60 days after infection. Figure D: At 90 days after infection. Figure E: At 180 days after infection. Figure F: control group

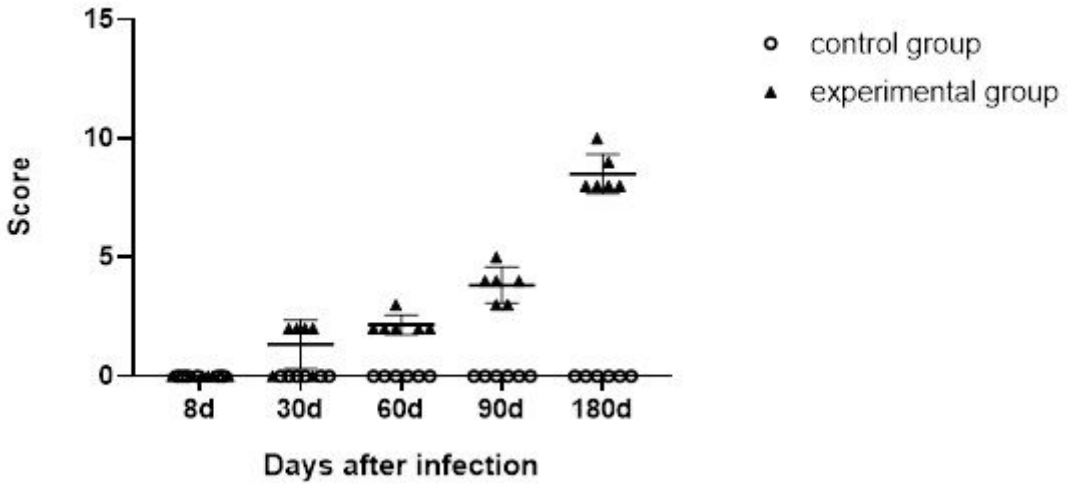

Figure 2

Vesicle growth score in mice infected with Echinococcus multilocularis

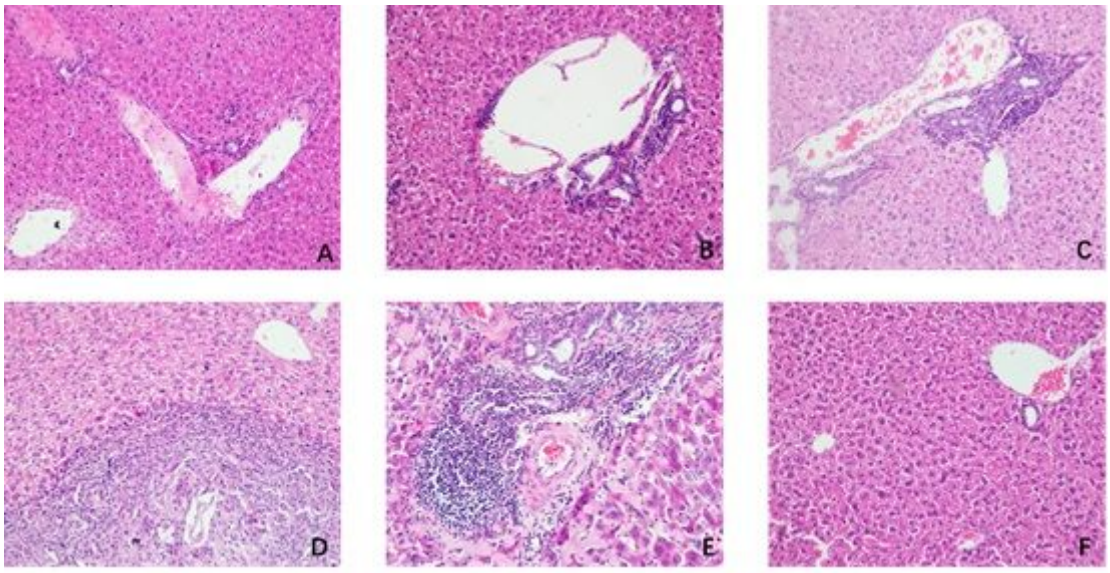

Figure 3

Pathological changes of mice liver during different infection stages (HE staining,200X) Figure A: At 8 days after infection. Figure B: At 30 days after infection. Figure C: At 60 days after infection. Figure D: At 90 days after infection. Figure E: At 180 days after infection. Figure F: control group

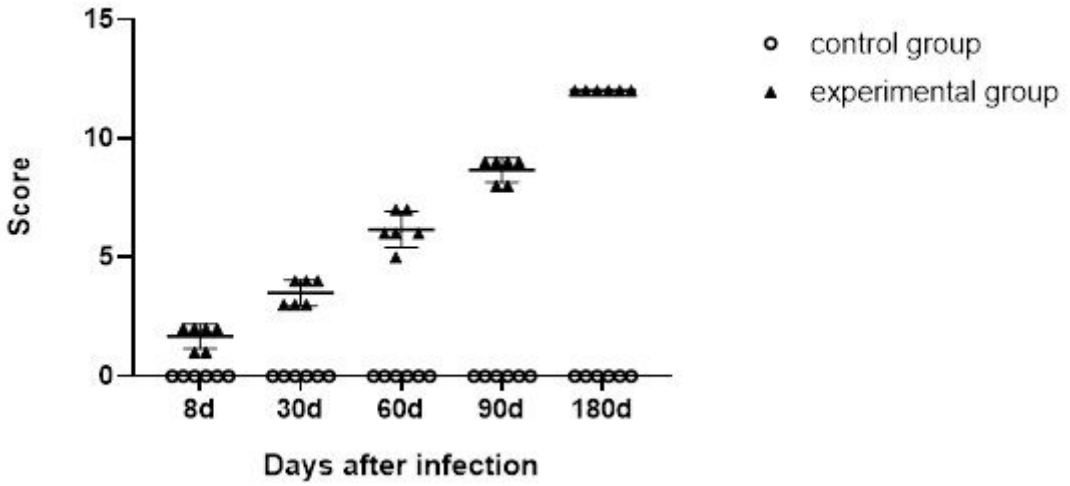

Figure 4

Histopathological Score of Liver in Mice Infected with Echinococcus multilocularis

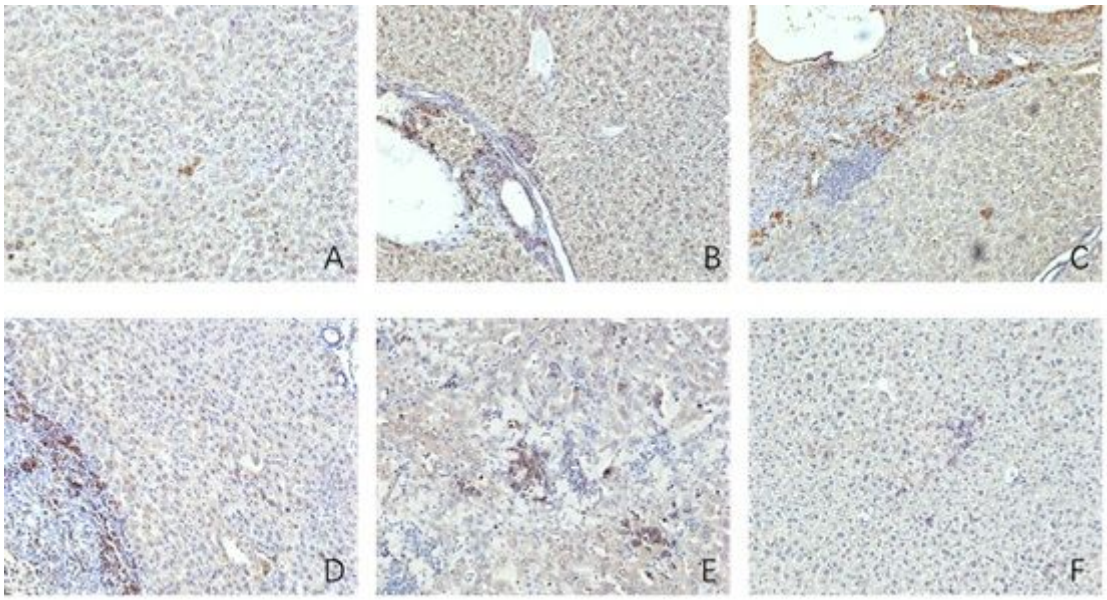

Figure 5

Expression and distribution of Beclin1 in different stages of infection (immunohistochemical staining, 200X) Figure A: At 8 days after infection. Figure B: At 30 days after infection. Figure C: At 60 days after infection. Figure D: At 90 days after infection. Figure E: At 180 days after infection. Figure F: control group

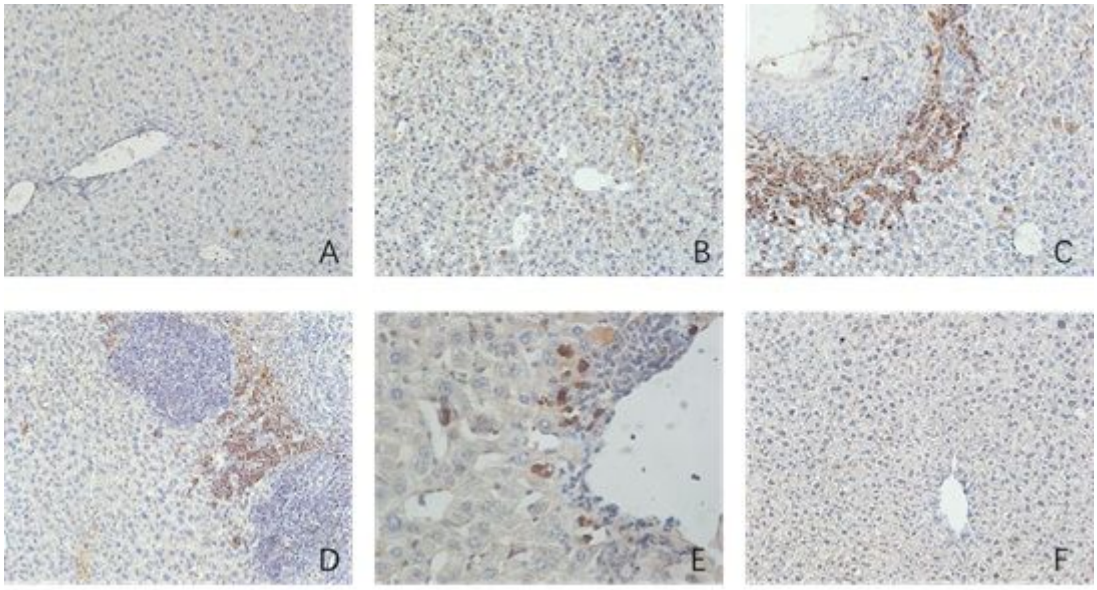

Figure 6

Expression and distribution of LC3 in different stages of infection (immunohistochemical staining, 200X) Figure A: At 8 days after infection. Figure B: At 30 days after infection. Figure C: At 60 days after infection. Figure D: At 90 days after infection. Figure E: At 180 days after infection. Figure F: control group

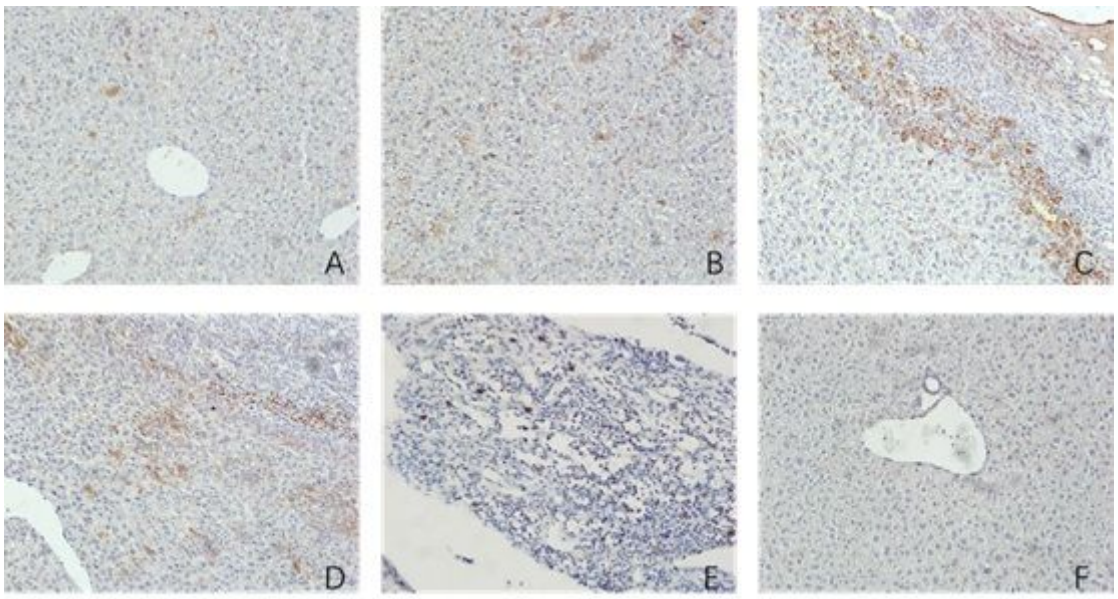

Figure 7

Expression and distribution of p62 in different stages of infection (immunohistochemical staining, 200X) Figure A: At 8 days after infection. Figure B: At 30 days after infection. Figure C: At 60 days after infection. Figure D: At 90 days after infection. Figure E: At 180 days after infection. Figure F: control group

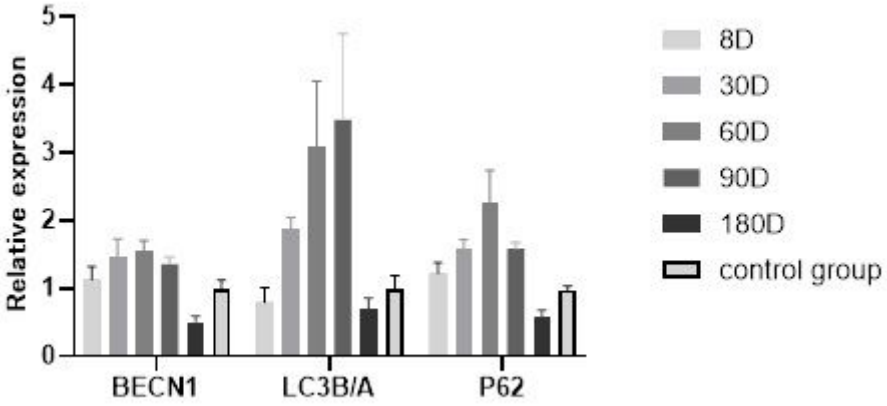

Figure 8

mRNA expression of autophagy-related proteins in mice liver at different stages of infection

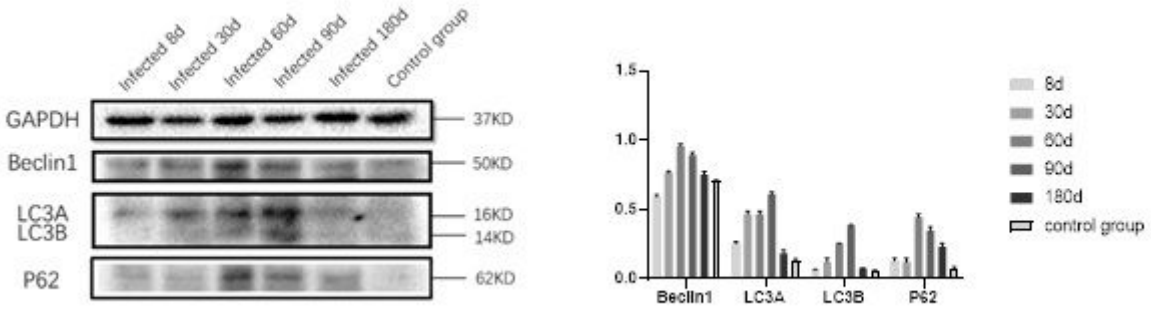

Figure 9

Expression level of autophagy-related protein at different stages in mice liver at different stages of infection