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Faculty of Chemical and Biopharmaceutical Technologies
Department of Industrial Pharmacy

Master's thesis

on the topic

RESEARCH OF ANTIPHLOGISTIC AND ANALGESIC PROPERTIES OF *American ginseng*

Completed: student of the group MPhch-20
of the speciality 226 Pharmacy, industrial pharmacy
(code and title of the specialty)
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ASSIGNMENTS
FOR THE MASTER'S THESIS

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1. Thesis topic Research of antiphlogistic and analgesic properties of *American ginseng*ng

2. Scientific supervisor Olha Nikitina, Associate Professor, PhD _____
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4. Initial data for work: scientific and information sources, methodological and technological literature, scientific periodicals, international and domestic regulations and standards for the development and production of medicines

5. Content of the thesis (list of questions to be developed). *a review of the literature on efficacy and research progress of American ginseng and Zebrafish and its application in anti- inflammatory field, Zebrafish was used as activity evaluation model, 3dpf macrophage green fluorescence transgenic zebrafish Tg (zlyz: EGFP) was used to construct inflammation model, LPS inflammation model, tail termination inflammation model and IBD inflammation model were constructed to evaluate the antiphlogisticactivity of American ginseng.*

6.Consultants of the master's thesis sections

Section	Surname, initials and position of the consultant	Signature	
		the task wasissued	the task accepted
Section 1	Olha Nikitina, Associate Professor		
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Section 3	Olha Nikitina, Associate Professor		

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Execution schedule

No	The name of the stages of the master's thesis	Terms of performance of stages	Note on performance
1	Introduction	20.09 –27.09.2021	
2	Section 1 *Research Progress on pharmacological activity of American ginseng*	28.09. – 11.10.2021	
3	Section 2 *Study on antiphlogistic and analgesic of American ginseng based on zebrafish model*	12.10 – 25.10.2021	
4	Section 3 *Study on industrialization of sliced American ginseng*	26.10 - 08.11.2021	
5	Conclusions	09.11.-15.11.2021	
6	Draw up a master's thesis (*final version*)	16.11.-04.12.2021	
7	Submission of master's thesis to the departmentfor review (*14 days before the defence*)	06.12.-14.12.2021	
8	Checking the master's thesis for signs of plagiarism (*10 days before the defence*)	14.12-18.12.2021	
9	Submission of master's thesis to the master's department to check the implementation of theappendix to the individual curriculum (*10 daysbefore the defence*)	16.12-18.12.2021	
10	Submission of master's thesis for approval by the head of the department (*from 7 days before the defence*)	18.12-21.12.2021	

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SUMMARY

Hongyuan Zou. Research of antiphlogistic and analgesic properties of *American ginseng*ng.

Master's thesis in the specialty 226 Pharmacy, industrial pharmacy - Kyiv National University of Technologies and Design, Kyiv, 2021.

The antiphlogistic and analgesic of American ginseng was confirmed in a model organism of zebrafish, and the possibility of commercial production of fine powder of American ginseng roots, ground to the level of destruction of the plant cell wall, was studied.

Zebrafish were used as a model for assessing antiphlogistic activity. Next models were designed: an endotoxin lipopolysaccharide, phenylthiourea, 2,4,6-trinitrobenzene sulfonic acid, -induced inflammation model and model is the tail fin amputation model.

The zebrafish activity results showed that all of the aforementioned models of inflammation confirmed the antiphlogistic activity of *Panax quinquefolium* in a dose-dependent manner within a safe dose.

Ultra-grinding of American ginseng roots has increased the release rate of the drug, improved dissolution of active pharmaceutical ingredients and improved absorption in the body, providing a solid theoretical basis and support for the development of industrial research.

Key words: *American ginseng, zebrafish, antiphlogistict, ultra-grinding, industrial research.*

АНОТАЦІЯ

Хунюань Цзоу. Дослідження протизапальних та знеболюючих властивостей *American ginseng*ng.

Дипломна магістерська робота за спеціальністю 226 Фармація, промислова фармація – Київський національний університет технологій та дизайну,Київ,2021р.

Протизапальну активність і знеболюючу активність американського женьшеню було підтверджено на модельному організмі рибок даніо. Вивчено можливість промислового виробництва дрібнодисперсного порошку коренів американського женьшеню, з руйнуванням рослинної клітинної стінки.

Рибок даніо використовували як модель для оцінки протизапальної активності. Були індуковані моделі запалення з ліпополісахаридом ендотоксину, фенілтіомочевиною, 2,4,6-тринітробензолсульфоновою кислотою і модель індукованого запалення після ампутації хвостового плавцю (травматична).

Результати дослідження активності рибок даніо показали, що на всіх вищезгаданих моделі запалення підтвердили протизапальну активність *Panax quinquefolium* дозозалежним чином у межах безпечної дози.

Ультраподрібнення коренів американського женьшеню збільшує швидкість вивільнення лікарської речовини, покращує розчинення активних фармацевтичних інгредієнтів та сприяє їх всмоктуванню в організмі, забезпечуючи міцну теоретичну основу та підтримку для розвитку промислових досліджень.

Ключові слова: *американський женьшень, даніо, протизапальний засіб, ультраподрібнення, промислові дослідження*.

ABBREVIATION LIST

IBD - inflammatory bowel disease

TCM - Traditional Chinese medicine

LPS - lipopolysaccharide

PTU - phenylthiourea

TNBS - 2,4,6-trinitrobenzene sulfonic acid

AGE - American ginseng extract

TNF - Tumor necrosis factor

IL-10 - Interleukin 10

IBD - inflammatory bowel diseases

qPCR - quantitative Polymerase chain reaction

NK - natural killer

PQS - total saponins of American ginseng stem and leaf and total saponins of Panax

PGs - ginseng stem and leaf

DNA - Deoxyribonucleic acid

MDA - malondialdehyde

SOD - dismutase

GSH PX - glutathione peroxidase

ZDV - azidothymidine

STZ - streptozotocin

FFA - free fatty acid

ISI - insulin sensitivity index

iNOS - Inducible nitric oxide synthase

COX - cyclooxygenase

PEG2 - Prostaglandin E2

OECD - Organisation for Economic Co-operation and Development

CD - Crohn's disease

UC - ulcerative colitis

TLC - Thin Layer Qualitative Identification

GC - gas chromatography

CONTENT

Introduction

The **relevance of the topic**. The use of *Panax ginseng* and *Panax quinquefolius* in traditional Chinese medicine dates back to about 5000 years ago thanks to its several beneficial and healing properties. Over the past few years, extensive preclinical and clinical evidence in the scientific literature worldwide has supported the beneficial effects of P. ginseng and *P. quinquefolius* in significant central nervous system, metabolic, infectious and neoplastic diseases. The world's largest producer of ginseng is China (44.749 tons), followed by South Korea (27.480 tons), Canada (6486 tons) and the United States (1054 tons). Data collected in 2009 confirm that Hong Kong is the biggest importer of ginseng root, whereas Canada is the biggest exporter in the world. As far as the market distribution is concerned, South Korea is the largest in the world; however, in this Country the domestic consumption of ginseng is larger than the amount exported. There has been growing research on ginseng because of its favorable pharmacokinetics, including the intestinal biotransformation which is responsible for the processing of ginsenosides - contained in the roots or extracts of ginseng - into metabolites with high pharmacological activity and how such principles act on numerous cell targets.

Dysregulation of the inflammatory response in humans can lead to various inflammatory diseases, like asthma and rheumatoid arthritis. The innate branch of the immune system, including macrophage and neutrophil functions, plays a critical role in all inflammatory diseases. This part of the immune system is well-conserved between humans and the zebrafish, which has emerged as a powerful animal model

for inflammation, because it offers the possibility to image and study inflammatory responses in vivo at the early life stages.

The **purpose of the study** is: determination of antiphlogistic activity of American ginseng extracts on model organisms of zebrafish and providing a theoretical basis for ultra-grinding of medicinal plant materials for traditional Chinese medicine.

The **research objectives of the study:**

- to analyze the mechanisms of the pharmacological action of biologically active compounds of American ginseng extract in inflammatory diseases of various etiologies;

- to discusses the various models of inflammation developed in zebrafish and how they are being used to study fundamental aspects of the inflammatory response and has also been used to screen libraries of natural compounds;

- to provide epidemiological characteristics inflammatory bowel disease (IBD);

- to determine the antiphlogistic activity of American ginseng extract in various models of inflammation induced in zebrafish;

- to substantiate the technology of grinding American ginseng medicinal plant materials at the level of destruction of the plant cell wall for the complete extraction of biologically active substances for use in TCM.

The **object of MTh** is are American ginseng extract and zebrafish whith models of inflammation an endotoxin LPS, PTU, TNBS, -induced inflammation model and model is the tail fin amputation model (traumatic).

The **subject of MTh** is the search for a safe and effective drug for the treatment of inflammatory bowel disease by determining the pharmacological action of American ginseng extract in a biological model of zebrafish.

Research methods: Transgenic Tg zebrafish containing green fluorescent 3dpf macrophages were used as a model to assess the activity. After egg collection, an inflammation inducer was added to suppress melanin production. When the zebrafish developed to 3dpf, healthy juveniles were selected and carefully added to each well. A blank control group (water prepared by the dilution system) and five concentrations of AGE were set. During cultivation, dead fish were removed. After three days of development, anesthetized young zebrafish were fixed on a glass slide in a lateral position using methylcellulose and their morphology was observed.

Practical value is the results of scientific research can be used for the development of drugs based on American ginseng extracts for the treatment of inflammatory bowel diseases, as well as the introduction into industrial production of ultra-crushed medicinal plant materials for use in TCM.

Elements of scientific novelty. For the first time, the antiphlogistic effect of American ginseng extract on an inflammatory model using zebrafish as a biological test object was shown.

Section 1. Research Progress on pharmacological activity of American ginse

1.1. Biologically active components and pharmacological action of American ginseng extracts.

There are eight species of ginseng in the world, seven in the genus *Panax* and one in the genus Eleutherococcus, the latter an Asian group of shrubs. Only three species, however, are widely used in herbal medicine, for which ginseng is widely known. These include *American ginseng* (*Panax quinque*folius), native to North America, *Oriental ginseng* (*P. ginseng*) native to Manchuria and Korea, and Siberian ginseng (*Eleutherococcus* [*Acanthopanax*]*senticosu*s), native to Siberia. *American ginseng* is a perennial herb of *Panax ginseng* in *Araliaceae*. It originally grows in the east of North America, mainly from Canada and the United States, and has been introduced and cultivated in China for many years [1,2,3]. Traditional Chinese medicine believes that American ginseng is cold in nature, which can replenish qi and nourish yin, clear fire and generate fluid. It is often used to treat Qi and yin deficiency, yin deficiency and fluid injury. Modern medical research shows that American ginseng has many effects such as improving immunity, anti-tumor, antiphlogisticand analgesic, anti-radiation and reducing blood sugar. It contains a variety of effective active components such as saponins, volatile oils, sugars and peptides 4.. American ginseng has strong antiphlogisticeffect. Its antiphlogisticeffect is mainly caused by reducing the secretion of NO and TNF by cytokines- α and IL-10, etc. When studying the effect of Tiepi Fengdou granule and its prescription drugs on chronic atrophic gastritis induced by nitrosoguanidine, long Huaqing and others found that *American ginseng extract*, as its prescription drugs, can also reduce body weight loss, gastric mucosal atrophy and intestinal metaplasia and achieve therapeutic effect [5]. Zhao Ying et al. Observed experimentally that the 20s protopanaxatriol saponins of *American ginseng* leaves may play a protective

role against cerebral ischemia-reperfusion injury in rats by inhibiting the production of inflammatory factors [6]. Liu Song et al. Established a rat cerebral ischemia-reperfusion model after giving *American ginseng* stem and leaf saponins to rats and detected the effect of plants stem and leaf saponins on TNF- α and IL-10 inflammatory cytokines, it is inferred that American ginseng stem and leaf saponin can slow down the inflammatory response, so as to effectively weaken the injury caused by cerebral ischemia-reperfusion [7]. Zhao Yali and others used American ginseng combined with Zuogui pill to observe the patients with chronic hepatitis C with liver and kidney deficiency in clinical Chinese medicine. It was found that the addition and subtraction of American ginseng Zuogui pill can improve pegylated interferon α- 2A induced neutropenia [8].

The main function of the digestive system is to absorb nutrients in food through mechanical chewing and enzymatic decomposition, and discharge metabolic waste out of the body. In addition, it also has strong secretion and immune functions, which can effectively prevent toxic substances, pathogenic microorganisms and toxins from entering the body and ensure the normal physiological function of the body [9]. As an important part of the digestive system, the intestine is rich in microorganisms, and the tight connection between cells provides strict barrier protection for the body. Based on this, once the microbial flora in the intestine is dysregulated or the immune barrier is destroyed, it will often lead to serious immune response. Such inflammatory reactions caused by intestinal mucosal injury and immune cell infiltration are collectively referred to as IBD. In the past, IBD incidence rate in western countries was higher. In 2016, there were more than 3

million IBD patients in the United States. The incidence rate of IBD in Asia is also increasing, and the degree of disease is similar to that in western countries, and gradually younger. In 2016, the International Conference on digestive diseases and digestive endoscopy in the South China, the famous IBD expert Edward V. Loftus pointed out that the incidence rate of IBD in China has reached 3.3/100000, which is second only to India (9.13/100000) [10] in Asia. The clinical manifestations of IBD patients mainly include diarrhea, bloody stool, abdominal pain, abdominal spasm, fever, fatigue, severe internal cramps in the pelvic area, muscle spasm, loss of appetite and weight loss. Because the pathogenesis of the disease is still in the exploratory stage, there is no effective treatment. Once the disease occurs, the patient will suffer from both physical and mental torture. Based on this, it is very important to establish an animal model of IBD disease by using appropriate model organisms and modern biological technology, and take this as the starting point to develop corresponding preventive and therapeutic drugs.

Zebrafish is a vertebrate. Compared with human genome, its gene has 70% homology. 82% of human disease-related genes can find homologous genes in zebrafish, and the whole genome similarity is as high as 87% [11]. Within one week after fertilization, zebrafish embryos can absorb the nutrients of yolk sac to maintain the nutritional needs of the body without feeding. At the same time, zebrafish in embryonic stage is transparent and the development of different organs can be observed at any time under the microscope. This feature can eliminate the impact of food on the research and is conducive to experimental research. Zebrafish has the characteristics of small volume, rapid reproduction, transparent embryo,

short experimental cycle, highly conservative genes with humans, highly similar physiological structure and biological characteristics with mammals, and conforms to the 3R (substitution, reduction and Optimization) principle of animal models [12,13]. Since 1994, zebrafish, one of the vertebrate models, has been gradually recognized by the academic community and widely used in the fields of genetics, developmental biology, mechanism research of major human diseases, drug development and safety evaluation [14,15]. Zebrafish immune system is highly conservative and has a complete reaction system such as inflammatory cell migration and phagocytosis similar to human [16][. Therefore, it is reliable and stable to study the antiphlogisticeffect of drugs by zebrafish model. At present, zebrafish inflammatory immune models mainly include traumatic inflammation model, drug-induced inflammation model, bacterial infection model and virus infection model [17-23].

The transgenic zebrafish TG (zlyz: DsRed) line with red fluorescence labeled inflammatory cells was used in this experiment. Direct observation of inflammatory response in vivo. The zebrafish inflammation model was established by tail injury and LPS induction to investigate the antiphlogisticand analgesic effects and corresponding mechanisms of American ginseng, and lay a foundation for the follow-up study on the mechanism of American ginseng in alleviating IBD. TNBS induction has the advantages of short modeling time, high repeatability and easy induction. It is a classical chemical mutagen for IBD [24]. TNBS can cause disappearance of intestinal peristalsis, intestinal dilatation and formation of intestinal obstruction in zebrafish. At the same time, it can also shorten the length of

intestinal villi and increase the number of goblet cells, which is very similar to the pathological manifestations of IBD patients [25]. The antiphlogisticmechanism of *American ginseng* was studied through the IBD inflammatory model induced by TNBS. Therefore, based on the previous work of our research group, focusing on the main antiphlogistic effects of *American ginseng*, this study uses the construction of zebrafish model to evaluate the antiphlogistic activity, and uses qPCR to determine the transcription level of zebrafish inflammation related genes in each group, so as to finally realize the research on the antiphlogistic mechanism of *American ginseng*. Based on the traditional efficacy of American ginseng, this study uses zebrafish disease model and qPCR to detect the transcription of related genes as research strategies, provides new methods and ideas for the drug action mechanism of complex Traditional Chinese medicine system, and also provides reference for the construction of *American ginseng* quality system.

American ginseng Linn. is a perennial herb of ginseng in *Araliaceae*. It originally grows in the east of North America, mainly from Canada and the United States, and has been introduced and cultivated in China for many years [26-28]. It is sweet, slightly bitter and cool in nature. It returns to the heart, lung and kidney meridians. In the clinical treatment of traditional Chinese medicine, *American ginseng* has the effects of Tonifying Qi and nourishing Yin, clearing heat and generating fluid. It is mainly used for the treatment of "deficiency of Qi and blood Yin, deficiency of heat and fatigue, expectoration, phlegm and blood, internal heat and thirst, dry mouth and dry throat" [29]. The chemical components of American

ginseng include ginsenoside, polysaccharide, volatile oil, organic acid, sterol, polyacetylene, amino acid, protein, etc. [30].

American ginseng has strong antiphlogisticeffect. Its antiphlogisticeffect is mainly caused by reducing the secretion of nitric oxide (no) and TNF by cytokines-α And IL-10, etc. When studying the effect of Tiepi Fengdou granule and its prescription drugs on chronic atrophic gastritis induced by nitrosoguanidine, long Huaqing and others found that *American ginseng* extract (AGE), as its prescription drugs, can also reduce body weight loss, gastric mucosal atrophy and intestinal metaplasia and achieve therapeutic effect [31]. Zhao Ying et al. Observed experimentally that the 20s protopanaxatriol saponins of *American ginseng* leaves may play a protective role against cerebral ischemia-reperfusion injury in rats by inhibiting the production of inflammatory factors [32]. Liu Song et al. Established a rat cerebral ischemia-reperfusion model after giving *American ginseng* stem and leaf saponins to rats, and detected the effect of plant stem and leaf saponins on TNF-α. And IL-10 inflammatory cytokines, it is inferred that American ginseng stem and leaf saponin can slow down the inflammatory response, so a.s to effectively weaken the injury caused by cerebral ischemia-reperfusion [33]. Zhao Yali and others used American ginseng combined with Zuogui pill to observe the patients with chronic hepatitis C with liver and kidney deficiency in clinical Chinese medicine. It was found that the addition and subtraction of American ginseng Zuogui pill can improve pegylated interferon α- 2A induced neutropenia [34].

The stems, leaves and fruits of *American ginseng* belong to its aboveground parts. Modern research has proved that the aboveground parts of *American ginseng*

contain a variety of active components mainly ginsenosides, and it has been proved that the content of total saponins in stems and leaves is significantly higher than that in roots, and the species and content of monomer saponins in stems, leaves and roots are also different 35-36.. Ginsenoside has a wide range of physiological activities. Laura L. Murphy et al. Proved that ginsenoside R is an anticancer active ingredient [37]. Panaxadiol has strong killing effect on green monkey kidney cancer. At high mass concentration, the inhibition rate of Panaxadiol on tumor is 55% [38].

Propanediol has strong inhibitory effect on the growth of breast cancer, lung cancer, prostate cancer and pancreatic cancer [39], Rosemary B.Duda and so on show that American ginseng has adjuvant effect on thymic cancer [40], because the total saponins extract has obvious anti-tumor effect, some saponins are used as anticancer drugs in clinical application. Polysaccharides in *American ginseng* are a kind of substances with special biological activities. At present, the isolated components include sucrose, ginseng trisaccharide, maltose, glucose, fructose, sorbose, galacturonic acid, galactose, glucose, arabinose, xylose, rhamnose, etc. Ma Xiuli et al. Isolated *American ginsen*g polysaccharide and studied the effect of the extracted polysaccharide on the growth of hepatoma cells in vitro. It was confirmed that the extracted polysaccharide could inhibit the growth of *7721 hepatoma* cells and promote their death [41]. *American ginseng* root polysaccharide can inhibit the tumor growth of S180 tumor bearing mice and significantly induce spleen lymphocytes to synthesize IL-3 like active substances [42]. Zhu Wenjing et al. Established the *BABL/ctjx* mouse liver cancer model, interfered with the occurrence and development of mouse liver cancer with polysaccharide, and observed the

changes of mouse liver tumor and spleen. The results showed that with the increase of polysaccharide dosage, the tumor mass of mice decreased significantly and the tumor inhibition rate increased significantly, indicating that American ginseng polysaccharide had an obvious inhibitory effect on tumor [43].

Immunity is a self-defense ability of the body. It includes the ability to recognize and eliminate foreign bodies and deal with adverse changes of the body. Therefore, whether it can improve immunity has become one of the focuses of drug research. In the experimental study, the evaluation of immune function mainly includes four indexes: cellular immunity, humoral immunity, monocyte macrophage function and NK cell activity. *American ginseng*, as a TCM for tonifying Qi, can supplement the material loss of human body and enhance human function, so as to improve disease resistance [44]. Liu Ying et al. Degraded diol ginsenoside in *American ginseng* leaves into 20 (s) - Ginsenoside Rg3 and 20 (R) - Ginsenoside Rg3 by acetic acid solution, and found that 20 (s) - Ginsenoside Rg3 and 20 (R) - Ginsenoside Rg3 could regulate Th1/Th2 immune imbalance in mice, providing a new scientific basis for the application of Ginsenoside Rg3 in immune system diseases [45]. By studying the effect of American ginseng flower polysaccharide on cell phagocytosis, Liu Xueying and others found that American ginseng flower polysaccharide can enhance macrophage immune activity, mainly by enhancing macrophage phagocytosis and releasing immune factors [46]. Zou Siying and others combined *Dendrobium candidum*, *American ginseng* and *Ganoderma lucidum*. After intragastric administration for 30 days, they carried out cellular immune experiment. The experiment mainly included delayed allergic reaction test and

mouse lymphocyte transformation test. Both experimental results were positive; The results of humoral immunity test, including antibody producing cell test and serum hemolysin test, were also positive. It is proved that this compatibility can effectively enhance the immune level of mice. At the same time, it also suggests that *American ginseng* can improve immunity in the compatibility of TCM [47]. Zou Shengcan and others found that the compatibility of American ginseng and medlar can enhance the immune function of immunocompromised mice [48]. Lu Zeyuan et al. Gave *American ginseng* and Schisandra chinensis formula to mice, observed the changes of a series of organ index, clearance index and phagocytosis index, found that *American ginseng Schisandra* formula can promote the production of serum hemolysin, T-lymphocyte proliferation and NK cell activity, and then inferred that the compatibility of *American ginseng* and *Schisandra chinensis* can enhance the specific and non-specific immune function of mice [49]. *American ginseng* stem and leaf saponins can promote the metabolism of peritoneal macrophages in mice, thereby enhancing the phagocytosis of peritoneal macrophages, and induce the production of nitric oxide in peritoneal cells of mice, indicating that plant stem and leaf saponins can activate macrophages, enhance the phagocytosis of macrophages, and produce bioactive substances, so as to enhance the immune function of the body [50]. Li Yan et al. Studied the regulatory effect of *American ginseng* root crude polysaccharide on the low immune function of mice caused by cyclophosphamide and found that medicinal herbal raw materials crude polysaccharide can significantly antagonize the reduction of leukocyte number and immune organ weight caused by cyclophosphamide, suggesting that *American ginseng*

polysaccharide can enhance the nonspecific immune and cellular immune function of the body, and increase with the increase of dose [51]. Lemmon et al separated *American ginseng* polysaccharides by ultrafiltration and studied the immune activity of each relative molecular weight segment. The results showed that plants polysaccharides with macromolecular mass played a key role in immune regulation [52]. Li Ji and others observed the anti-fatigue effect of *American ginseng*, the effect on the intensity of delayed type hypersensitivity and the function of mononuclear phagocytes. Results *American ginseng* could significantly improve the immune ability of yin deficiency mice induced by hydrocortisone; Improve the intensity of delayed type hypersensitivity and the ability of mononuclear phagocytes in mice [53]. It shows that the drug can improve the body's immunity and strengthen the foundation. Chen Qin et al. Used mitotic index test, chromosome aberration test and micronucleus test of mouse bone marrow lymphocytes to observe the effect of American ginseng on genetic damage of mouse bone marrow lymphocytes. It is suggested that American ginseng decoction can repair or protect the chromosome damage of mouse bone marrow cells induced by mitomycin [54]. Batu et al. Studied the effects of total PQS and total PGs on DNA damage in testis and spleen of BALB/c mice induced by cyclophosphamide with inhibition of DNA synthesis as an index. The results showed that PQS and PGs had obvious anti-DNA damage effects [55].

Free radicals are the intermediate products of biochemical reactions in the process of human life activities. Under normal circumstances, the production and elimination of free radicals in the body are in dynamic balance, but if the production

of free radicals in the body is too much or the elimination is too slow, the free radicals will cause damage to the body at the molecular level, cell level and organ level. Zheng Chaohua et al. Studied the extraction of total flavonoids from *American ginseng* and its effect on hydroxyl radical scavenging. The results showed that the extract of *American ginseng* contained effective components with hydroxyl radical (-OH) scavenging ability and had a certain scavenging effect 56.. Wu Huazhang et al. Discussed the antioxidant function of *American ginseng* saponin and its protective effect on genetic damage in mice caused by cyclophosphamide. *American ginseng* saponin has strong antioxidant activity in vivo and in vitro and has obvious protective effect on genetic damage in mice caused by cyclophosphamide. Its mechanism may be related to improving the antioxidant capacity of the body and enhancing the anti mutagenic capacity of mice [57]. Studies have shown that *American ginseng* polysaccharide peptide can reduce the content of MDA in serum and improve the activities of SOD and GSH PX, so as to play an antioxidant role [58]. AGE can induce the activity of phase II metabolic enzyme quinone reductase and has antioxidant activity in vitro. Its induction of phase II metabolic enzyme can cause its pharmacokinetic interaction with ZDV and abacavir, so as to reduce the plasma concentration of these drugs and increase the risk of treatment failure and drug resistance [59]. American ginseng oral liquid has a good scavenging effect on sodium nitrite, can significantly improve the activity of plasma antioxidant dismutase and reduce the content of malondialdehyde [60]. *American ginseng* stem and leaf saponins can significantly reduce the content of malondialdehyde in whole blood and myocardial tissue of rats induced by adriamycin, protect the activities of

superoxide dismutase and glutathione peroxidase, indicating that American ginseng stem and leaf saponins have antioxidant effect [61].

Yin Huijun and others observed the effects of *American ginseng* saponins on blood glucose, blood lipid and serum insulin levels in alloxan hyperglycemic rats. The results showed that plants saponins could significantly reduce the levels of blood glucose, serum total cholesterol and triglyceride in hyperglycemic rats, and increase the contents of serum high density lipoprotein and insulin [62]. Diabetic mice were induced by alloxan, and the mice were fed with different dosages of *American ginseng* polysaccharide peptide. The body weight, fasting blood glucose, glucose tolerance, blood lipid level and serum antioxidant capacity in each experimental group were observed in four groups. The results showed that plants polysaccharide peptide had the effects of reducing blood glucose, regulating lipid metabolism and anti lipid peroxidation [63]. Zhang Ying et al. Observed the effects of PQS on glucose and lipid metabolism and insulin resistance signal transduction of adipocytes. The results showed that total PQS could promote glucose utilization by adipocytes and inhibit the pro lipolysis of TNF, so as to regulate glucose and lipid metabolism [64]. Ge Pengling et al. Used high-fat diet feeding combined with one-time low-dose injection of STZ to replicate the rat model of insulin resistance and observed the effects of American ginseng on the levels of triglyceride, total cholesterol, FFA and ISI. Results after the intervention of *American ginseng*, the insulin sensitivity index increased and the levels of triglyceride, total cholesterol and free fatty acid decreased. It shows that *American ginseng* can significantly improve the abnormal lipid metabolism in insulin resistant rats [65].

Guo Chunyu et al. Studied the protective effect of PQS on non infarcted tissue in rats with acute myocardial infarction. The results showed that total saponins of stem and leaf could protect the damaged non ischemic myocardial tissue after myocardial infarction through antiphlogistic, protecting vascular endothelium and regulating energy metabolism [66]. Lu Meijun and others observed the effect of PQS on S-100 in serum of rats with cerebral ischemia β It was found that stem and leaf saponins could improve the symptoms of neurological deficit and reduce S-100 in blood β The content of protein has a protective effect on cerebral ischemia in rats [67]. *American ginseng* saponins can reduce hypoxia / reoxygenation injury of isolated rat cardiomyocytes and ischemia-reperfusion injury of rats by inhibiting endoplasmic reticulum stress-related apoptosis, and reduce ventricular remodeling after acute myocardial infarction [68]. Studies have shown that *American ginseng* diol saponins protect experimental myocardial ischemia by blocking calcium channels, reducing oxidative damage of free radicals to myocardium and inhibiting the production of angiotensin II in acute myocardial infarction [69]. After myocardial infarction was caused by ligation of the anterior descending branch of the left coronary artery in rats for 4 weeks, the experiment of ventricular remodeling model confirmed that diol saponins had a preventive and therapeutic effect on ventricular remodeling caused by myocardial infarction [70]. *American ginsen*g diol saponins could significantly reduce the contents of angiotensin II and endothelin in plasma and myocardium, Its effect is consistent with benazepril, an angiotensin converting enzyme inhibitor for clinical prevention and treatment of ventricular remodeling [71].

In addition to the above effects, American ginseng also has other pharmacological effects. Feng kunmiao et al. Extracted polysaccharides from the stems and leaves of *American ginseng* and carried out anti-virus experiments with their polysaccharides. The results showed that the polysaccharide extract had obvious anti-virus effect [72]. In general, modern experiments show that *American gin*seng has significant effects on cardiovascular system, anti-tumor, immune regulation, antioxidant and so on. At the same time, the role of *American ginseng* in other aspects also provides a theoretical basis for the development of *American ginseng* as medicine.

1.2. Zebrafish and its application in antiphlogisticfield

Zebrafish is a tropical freshwater ornamental fish native to South Asia. It is named for its longitudinal blue and silver stripes like zebra. In the 1970s and 1980s, American molecular biologist Streisinger and his colleagues took the lead in using zebrafish as experimental animals [73]. At present, zebrafish has been widely used in developmental biology, oncology, toxicology, genetics, neurobiology and other fields [49-52], and is considered to be an ideal model for studying development, immunity, physiology, nutrition, genetics and behavior [74-76].

Compared with other model animals, zebrafish species are stable and individual differences are small; Small size, strong resistance to pathogens, easy to raise on a large scale, and low strain maintenance cost; Strong reproductive ability and large number of eggs, which can realize positive genetic research based on phenotype; The breeding cycle is short, which can significantly shorten the research cycle. Compared with other fish, zebrafish eggs are larger, embryos develop in vitro

and transparent, which is convenient for embryo operation and imaging; The organ development is very similar to that of mammals; It has easy to observe and test developmental behavior, and is suitable for morphological observation and anatomical research. Zebrafish genome has been completely sequenced and has 70% ~ 80% similarity with human genome [77]; Zebrafish is easier to carry out genetic operation than other vertebrate models (such as mice). Gene editing technology and various cell biological technologies have been developed in zebrafish model, which is conducive to the study of gene function in zebrafish; At the same time, the establishment and development of a large number of zebrafish genetic strains provide great convenience for the study of zebrafish model.

The immune system of zebrafish is highly conservative. Its immune cell type and morphology are similar to human beings, including neutrophils, monocytes, macrophages, lymphocytes and so on. Zebrafish adult fish, like mammals, have natural immune system and acquired immune system. Cells of natural immune system (neutrophils and macrophages) first appear and develop acquired immunity 3 ~ 4 weeks after fertilization [78]. Zebrafish has a rapid natural immune response to infection and tissue damage, and is easy to induce inflammatory response, making it an ideal system for studying inflammation and wound repair [79]. The establishment and development of completely transparent zebrafish embryos in the first 2 weeks after birth and transgenic zebrafish strains labeled by immune cell fluorescence make it feasible to track and observe the immune response in zebrafish embryos, a complete organism [80].

Inflammation models commonly used in zebrafish. There are many inducing factors of inflammation, which can be mechanical injury, bacterial and viral infection, or chemical induction. At present, zebrafish experiments basically use the following three methods to simulate the stress process of immune system for inflammation, namely local inflammation induced by tail amputation, systemic inflammation induced by LPS and acute inflammation induced by copper sulfate (CuSO4).

Zebrafish tail amputation inflammation model is a traumatic inflammation model. The treatment of zebrafish tail amputation can induce local damage to zebrafish tail and promote immune response of zebrafish immune cells [81]. In the early stage of zebrafish embryonic development, macrophages and neutrophils participate in the inflammatory response together. The number of macrophages and neutrophils in the wound reaches the peak 6 hours after zebrafish tail amputation, and the inflammatory response begins to subside 6 hours later [82,83]. Researchers can study the inflammatory mechanism and evaluate the antiphlogisticactivity of drugs by describing the migration, aggregation and regeneration of immune cells after injury.

Zebrafish LPS inflammation model is a systemic inflammation model induced by LPS. LPS is the main structural component of the cell wall of Gram-negative bacteria. It is an endotoxin. It is one of the main ligands recognized by the innate immune system through pattern recognition receptors. It can cause inflammatory cascade [84]. It is a common inducer of mammalian inflammatory model and is also widely used in zebrafish inflammatory model. LPS immersion administration or

yolk sac microinjection administration of zebrafish embryos 2 ~ 3 days after fertilization can induce an increase in the number of zebrafish immune cells and induce inflammatory response [85]. Whether drug treatment can reverse the increase of immune cells caused by LPS has become one of the important indexes for the evaluation of its antiphlogisticactivity.

Zebrafish CuSO4 inflammation model is an acute inflammation model induced by CuSO4. Copper is a functional component of the innate immune system. It induces oxidative stress through ROS and actively regulates inflammatory response. In juvenile and adult zebrafish, CuSO4 stimulation can rapidly migrate zebrafish immune cells to the neurocolliculus. Therefore, CuSO4 exposure is often used to induce and simulate inflammatory characteristics [86]. Different from the methods of physical injury and infectious agent, copper as an inflammatory agent can be operated by non-invasive method. The first mock exam is to prevent the migration of immune cells to the neural hillock and to reflux immune cells.

Inflammatory mediators in zebrafish model. In the process of zebrafish inflammatory response, after immune cells are activated, many types of soluble mediators will be produced, mainly cytokines, chemokines, complement system, lipid mediators, etc. these mediators will amplify the inflammatory response in the follow-up, eliminate invading pathogens or repair damaged tissues [87]. Due to the conservation of inflammatory mediators in zebrafish, zebrafish has become a potential animal model for inflammatory evaluation.

Cytokines are a kind of small molecular proteins synthesized and secreted by immune cells and some non-immune cells. They are the key regulators of

inflammation and participate in acute and chronic inflammation through complex signal pathways. Zebrafish cytokines are similar to mammals in structure and function. More and more studies use zebrafish inflammation model to evaluate the antiphlogisticactivity of traditional Chinese medicine by detecting the expression level of cytokines. Among many cytokines, interleukin-1 plays a major role β, IL-6, IL-8, IL-10, tumor necrosis factor- α (tumor necrosis factor- α, TNF- α), Interferon- γ (interferon- γ, IFN- γ), Transforming growth factor- β (transforming growth factor- β, TGF- β) Et al. 88,89.. Where IL-1 β, IL-6, IL-8, TNF- α, IFN- γ It can stimulate and maintain inflammatory response and is an important pro-inflammatory cytokine [34]; IL-10 and TGF- β. It is a key antiphlogisticcytokine involved in inflammatory response and immunosuppression [90,91]. Chen et al. 92. used zebrafish CuSO4 inflammatory model to study the antiphlogisticactivity of Gardenia extract, and detected IL-1 by real-time fluorescence quantitative PCR (rt-q PCR) β, IL-6 and TNF-α It was found that gardenia extract could significantly reduce the expression level of these cytokines, which confirmed the antiphlogisticactivity of Gardenia extract.

Chemokines are a group of inflammatory mediators indispensable for immune cell migration. They can play a role in inflammation and normal physiological conditions. Chemokines are defined based on their amino acid composition, including CXC, CC, C and CX3C [93,94]. In zebrafish, 89 chemokine genes and 33 chemokine receptors have been identified [95]. Among them, only cxcl12a, cxcl12b, cxcl19 and CXCL8 (IL-8) ligand receptor interactions are conserved with mammals [96]. At the same time, a large number of zebrafish

chemokines have not been identified, and the study of the genetic and biochemical characteristics of these chemokines will further expand the advantages of zebrafish models in the field of TCM antiphlogistic.

The complement system is a complex protein network involved in acute or chronic inflammatory response Complement deposition is often used as an immunohistochemical marker of inflammation. Many components and signal pathways of complement system in mammals are highly conserved in zebrafish [98]. Through the study of the development process and function of complement system in zebrafish early embryos, Li Zongyao et al. [99] found that 8 complement related genes (C3, C4, C9, BF1, BF2, BF3, rca2.1 and rca2.2) were detected before embryo development to pharyngeal embryo stage MiRNA is widely expressed in the whole embryo. After LPS stimulation, C3, C9, BF1, BF2 and BF3 genes were up regulated, rca2.1 genes were down regulated, and C4 and rca2.1 genes had no significant changes, suggesting that zebrafish can build complement system in the early embryonic development and participate in immune responses such as acute phase response.

Lipid mediators are widely involved in inflammatory response, mainly including prostaglandins, which can cause vasodilation and promote inflammation [100,101]. iNOS exists in macrophages and can produce active nitrogen free radicals. It is a catalytic enzyme that can reflect the degree of inflammation [102]. Macrophages and other cells stimulated by inflammation can produce an enzyme that can regulate prostaglandin synthesis - COX [103], and its isoenzyme COX-2, as an inducible enzyme, can participate in the process of inflammatory regulation.

PEG2 synthase (ptges) can be activated and regulated by iNOS and COX-2 to produce PEG2 in the process of inflammatory response [104]. Li et al. [105]. Using RAW264.7 cell line and zebrafish model to study the antiphlogisticactivity of red mushroom polysaccharide extract, it was found that rap could significantly reverse the up regulation of iNOS and COX-2 protein levels induced by LPS. Kwon et al. [106] studied the effect of mulberry leaf aqueous extract (wemf) on inflammatory mediators and found that wemf could significantly inhibit the secretion of PEG2.

As a new model organism, zebrafish has the advantages of small volume, strong reproductive ability, rapid development, easy observation and highly conservative immune system. With the continuous improvement of technology and methods, zebrafish has been gradually applied in the field of antiphlogisticof TCM in recent years. As a complete organism, zebrafish can objectively, comprehensively and systematically evaluate the antiphlogisticactivity of TCM and its effective components, realize rapid and effective high-throughput screening, and also provide a good model for the study of the mechanism of antiphlogistic TCM. The full use of zebrafish model will provide new ideas and methods for the research of antiphlogistic TCM and promote the development of antiphlogisticfield of TCM. At the same time, zebrafish model will also have a broader application prospect in the field of antiphlogistic TCM.

1.3. Research progress of inflammatory bowel disease

IBD is a chronic inflammatory bowel disease mainly involving the digestive system, in which CD and UC are the main types. In recent decades, with the process of industrialization and urbanization in non-developed countries and regions such as

Asia, South America and the Middle East, IBD has also begun to appear in newly industrialized countries. At present, IBD has become a global disease. According to the epidemiological data, the prevalence and incidence rate of IBD are increasing. It has become a common disease and frequently occurring disease in China. Many provinces have included IBD in outpatient chronic diseases. IBD is a chronic disease, which mainly involves the digestive tract, but also invades extraintestinal organs and tissues such as joints and eyes, and ultimately endangers human health and affects the quality of life of patients. However, at present, the etiology and pathogenesis of IBD are still unclear. Domestic attention and research institutions are also strengthening and deepening.

Epidemiological status of IBD. IBD was first found in western countries. So far, the etiology is unclear, the pathogenesis is complex, and there is a lack of clear mechanism. In the middle of twentieth Century, according to epidemiological survey data from developed countries and regions in Europe, North America, Australia and New Zealand, the incidence rate of UC and CD increased in the past decades, reaching the highest level in early twenty-first Century, and the highest incidence rate of CD in Canada, northern Europe, New Zealand and Australia were 20.2/10, 10.6/10, 16.5/10 and 29.3/10 respectively. The highest prevalence rates were 322 / 100000 in Europe, Canada and the United States, 319 / 100000 in Canada and 214 / 100000 in the United States. The highest incidence rate of UC in northern Europe, Canada and Australia is 24.3/10 million, 19.2/10 10000, 17.4/10 million; The highest prevalence rates were 505 / 100000 in Europe, 248 / 100000 in Canada and 214 / 100000 in the United States. At present, the number of patients in Europe

and America has accounted for 0.5% of the world's population. There are regional differences in incidence rate and prevalence rate. City areas are higher than rural areas. The age of onset of IBD is still dominated by adolescents and adults. Since 1990s, the number of children's IBD has gradually increased in western developed countries and regions. Until 2018, a systematic review of 140 studies showed that the incidence rate of IBD in China is increasing [107] in developed countries or developing countries. Studies have shown that the risk of immigrant populations from low incidence rate to high incidence rate is similar to that of non immigrant population, which indicates that genetic factors play a role in the pathogenesis of IBD. The latest statistics in 2018 show that over the past 20 years, the incidence and prevalence of IBD in western developed countries have begun to stabilize and have been in a plateau stage. The incidence rate and incidence rate of non-western countries are increasing obviously, especially in South America and East Asia [108-110].

The incidence of IBD in Asia also showed an upward trend. According to previous records, the incidence rate of CD in Japan was 0.51/10 million from the beginning of 90s, and the prevalence rate was 5.85/10 million, the incidence rate was 20~24 years for males and 15~19 years for females. The incidence rate of UC is 1.95/10 million, the prevalence rate is 18.12/10, the incidence rate is 20~24 years for males and 25~29 years for females. A latest study in 2016 can understand the epidemic data of IBD [111]: more than 200000 cases of IBD have been recorded in Japan since the 1970s, and the prevalence rate of CD is 18.6/100000; The prevalence of UC was 57.3/100000. Compared with previous data, the prevalence of IBD in

Japan showed an obvious upward trend. Epidemiological studies on IBD in children were also carried out earlier in Japan. The data show that the age of 10.6% CD and 5.9% UC is ≤ 16 years [112,113]. The latest data show that the prevalence rates of CD and UC in age adjusted children (0 ~ 19 years old) in 2004 were 4.2/100000 and 11.1/100000 respectively, and the corresponding prevalence rates of young people (20 ~ 39 years old) were 41.0/100000 and 89.8/100000 respectively. By 2016, the prevalence rates of CD and UC were 7.2/100000 and 15.0/100000 respectively. In comparison, the prevalence rate of UC was higher than that of CD [114].

Moreover, the proportion of spontaneous abortion and caesarean section of IBD pregnant women in Japan has increased significantly, and the birth rate of low-birth-weight infants has also increased significantly [115]. In early twentieth Century, a large sample epidemiological survey was conducted among IBD population in various regions of South Korea. According to the latest data, the incidence rate of CD in Korea was 3.2/10 and UC incidence rate was 4.6/10 [116]. According to the latest integrated epidemiology study of Crohn's disease and colitis in the Asia Pacific region, the average incidence rate of IBD in Asia in 2011 was 1.4/10 million, and the incidence rate of growth was increasing year by year, and UC was two times higher than that of CD, but the rate of CD growth was faster than UC[11]..

Before last century, there were occasional reports of IBD in Asian developing countries. With the transformation of developing countries and newly industrialized countries from agriculture to industrialization, urbanization has significantly changed people's lifestyle, diet, daily activities and disease exposure factors. With

the development of industrialization and urbanization, IBD in developing countries is reported more and more. Countries like India have an increasing incidence rate. The statistics show that UC is more common in China than [118]. Other Asian countries such as Sri Lanka, Indonesia, Thailand, Malaysia and [119] also have higher incidence rate and incidence rate than in the past, but they are still lower than those in Europe and the United States.

China's incidence rate and prevalence rate of IBD are increasing. According to the data of China Center for Disease Control and prevention in 2014, the total IBD cases in China are about 350 thousand in 2005-2014 years. By 2025, the number of IBD patients in China will reach 1 million 500 thousand [120].

From the preliminary epidemiological data analysis in China, we can find that there are many differences in the characteristics of IBD between Asia and Western countries. Because the disease has attracted more and more attention in recent 20 years; At present, grass-roots doctors do not have a high awareness of such diseases, and many patients delay diagnosis and treatment is common; At the same time, ordinary people have insufficient understanding of it, resulting in delayed diagnosis and treatment. Moreover, IBD has the characteristics of occult, diverse and nonspecific symptoms, unreported diagnosis, misdiagnosis or misclassification, long initial diagnosis time and low mortality, which also brings great challenges to epidemiological research.

Although there is a consensus on the diagnosis and treatment of IBD in China, they all refer to foreign diagnosis and treatment data, guidelines and consensus to a certain extent, combined with the opinions of domestic experts. To improve the

domestic IBD epidemiological data, we will establish our IBD data based on the IBD incidence rate, prevalence rate, high risk factors and clinical manifestations, which is helpful to discover the characteristics of IBD in China, and make targeted strategies for prevention, individualized chemotherapy and drug research and development.

In addition, China has entered an aging country. How to deal with the gradual aging of IBD population and study the epidemiological characteristics of IBD elderly population need to be improved. To understand the epidemiological big data of IBD in China, so as to provide better strategies for the diagnosis, treatment and prevention of IBD in China. Moreover, the epidemiological study of IBD children and pregnant women in China is still a blank, which needs to be solved and further improved.

Immunopathogenesis of inflammatory bowel disease. Th cells are T cells with auxiliary function and express CD4 molecules. It has been confirmed that IBD is an immune response process induced by cytokines produced by CD4 + Th cells 121.. The pathogenesis of CD is related to Th1 and Th17 cells, while UC is closely related to Th2 cells [121,122].

Th1 cells are one of the earliest Th cell subsets. IL-12 secreted by macrophages can stimulate antigen-presenting cells to secrete interferon- γ (interferon- γ, IFN- γ), Signal transducers and activators of transcription-1 (STAT-1) acting on initial CD4 + T cells, which transfers to the nucleus to reactivate the specific transcription factor T-bet and promote the differentiation of Th0 into Th1 123.- γ Activate monocyte macrophages to release tumor necrosis factor- α (tumor necrosis factor- α, TNF- α),

Proinflammatory cytokines such as IL-1 and IL-6 participate in the inflammatory process and form IFN- γ, TNF- α Elevated Th1 mucosal inflammation plays a key role in the pathogenesis of CD [124].

Th2 cells are also one of the earlier discovered Th cell subsets. Antigen presenting cells secrete IL-4 and activate STAT-6 after acting on the surface receptor of primitive Th cells. The latter transfers to the nucleus to activate specific transcription activator GATA-3, which makes Th0 cells differentiate into Th2 through downstream gene expression [125]. Th2 mainly secretes inflammatory factors such as IL-4, IL-5 and IL-13 to form Th2 mucosal inflammation. There is also evidence that the increased autoantibodies in UC are mainly Th2 related antibody types, so it is speculated that Th2 may play a key role in the pathogenesis of UC 126.. There is an atypical NK cell in the colonic mucosa of UC patients, which can promote the secretion of IL-13 by Th2 cells, which also shows the important role of Th2 in the pathogenesis of UC [127].

Th1 and Th2 cells alone and interact to regulate the immune balance of the body and maintain the relative stability of the internal environment. It is known that the dominant cytokines of Th1 cells are mainly IL-12 and IFN- γ, the dominant cytokines of Th2 cells are IL-4, IL-5 and IL-13 125,128.. It has been confirmed that the dominant cytokine IFN of IL-12 and Th1 cells- γ Both can promote the polarization of CD4 + T cells to Th1 cells and inhibit the polarization to Th2 cells, while the dominant cytokine IL-4 of Th2 cells can promote the polarization of CD4 + T cells to Th2 cells and inhibit the polarization to Th1 cells, regulating the immune balance of the body [124,127]. The imbalance of immune regulation of Th1 / Th2

subsets is related to the occurrence of autoimmune diseases and a variety of inflammatory diseases, especially in the occurrence and development of IBD [129].

Cytokines are low molecular weight soluble proteins produced by cells stimulated by immunogens, mitogens or other factors. Cytokines are bioinformatics factors, which can regulate innate and adaptive immune responses, stimulate cell activation, proliferation and differentiation. Proinflammatory cytokines such as IL-1, IL-2, IL-6, TNF- α, INF- γ and antiphlogisticcytokines such as IL-10, IL-35 and TGF- β the imbalance between is an important factor causing intestinal mucosal injury.

IL-1 is produced by monocyte macrophages, dendritic cells and epithelial cells, participates in the activation of T cells, NK cells and macrophages, and has a wide range of immunomodulatory effects. Ashwood et al. 130. found that the level of IL-1 in UC biopsy tissue increased and increased with the degree of inflammatory activity, which can be used as an index to judge the severity and curative effect of the disease.

IL-2 is mainly produced by Th1 cells, which is a T cell growth factor and enhances the killing activity of NK cells and macrophages. The level of IL-2 in intestinal mucosa is consistent with the degree of IBD inflammation. The lymphocytes of IBD patients are activated, and the IL-2 receptor in serum gradually increases with the aggravation of the disease [108]. IL-2 is an important pro-inflammatory cytokine. Interfering with IL-2 signaling can effectively prevent the occurrence of autoimmunity [131]. Clinical trials have confirmed that the treatment of refractory UC with anti-IL-2 receptor antibody can significantly

improve the patients' clinical symptoms, endoscopic manifestations, immunohistochemical results and quality of life, suggesting that IL-2 plays an important role in the pathogenesis of UC [132].

IL-6 is mainly produced by monocyte macrophages and Th2 cells. It is composed of IL-6 receptor (IL-6R) and signal transduction protein gp130. It can stimulate the growth and differentiation of T cells and B cells and promote the proliferation of a variety of cells. IL-6 can induce the polarized expression of intercellular adhesion molecule (ICAM) through Stat-3 pathway, which plays an important role in the interaction between neutrophils and epithelial cells in patients with IBD. Therefore, it is considered that IL-6 plays an important role in chronic intestinal inflammatory response. Studies have shown that the levels of IL-6 and IL-6R in peripheral blood of IBD patients, especially CD patients, are significantly increased and positively correlated with disease activity [133]. TNF is produced by macrophages, NK cells, T cells and other cells, which can improve the phagocytosis of neutrophils, increase the production of peroxide anion, stimulate cell degranulation and secrete myeloperoxidase. TNF can increase the expression of I antigen, ICAM-1 and the secretion of IL-1, granulocyte macrophage colony stimulating factor (GM-CSF) and IL-8, promote neutrophil adhesion to endothelial cells and stimulate local inflammatory response. TNF- α Specific TNF- β the function of is strong. TNF- α After neutrophils are activated, they induce the production of platelet activating factor (PAF), leukotriene B4 (LTB4), thromboxane A2 (TXA2), elastase, O2 and H2O2, change the anticoagulant activity in small vessels and cells of intestinal lamina propria, induce procoagulant activity and

inhibit thrombomodulin (TM), On the basis of small vasculitis of intestinal lamina propria in patients with IBD, micro thrombosis is formed, resulting in disturbance of intestinal mucosal microcirculation.

IL-10 is a typical antiphlogisticcytokine produced by Th2 cells and a cytokine with immunosuppressive effect [134]. It is a key cytokine to antagonize Th1 cells and has a direct inhibitory effect on Th1 cells. IL-10 can inhibit IL-2 and TNF- α and IFN- γ to prevent inflammation. IL-10 has a direct effect on macrophages and can limit IL-1, IL-6, IL-8 and TNF- α, IL-12 and granulocyte macrophage colony stimulating factor can restore tolerance to intestinal flora and reduce intestinal symptoms. IL-10 can protect lymphocytes that inhibit IBD, inhibit host autoimmune response mediated by IL-10, and have important anticancer effect 135.. Knockout of IL-10 gene is prone to autoimmune diseases in animals, including CD like colitis mediated by Th1 cells [135].

Section 2. Study on antiphlogistic activity of *American ginseng* based on zebrafish model

2.1. Materials and methods

Main reagent and sample information. Endotoxin lipopolysaccharide (LPS), phenylthiourea (PTU), Tricaine, methyl cellulose, 2,4,6-trinitrobenzene sulfonic acid (TNBS), calcein were purchased from sigma (St. Louis, Mo, USA); Methylene blue was purchased from Sinopharm Chemical Reagent Co., Ltd.

Fish culture water allocation method (5 mM NaCl, 0.17 mm KCl, 0.4 mM CaCl2, 0.16 mm MgSO4). American ginseng extract was prepared into 30 μg/ml, and then diluted to the working concentration during administration.

The characteristics of the instruments that were used in the experiment are presented in the table

Equipment name and Model Company

Equipment name and Model	Company
Zebrafish breeding system	Beijing Aisheng Technology Co., Ltd
Forma 3111 water jacket CO_2 incubator	Forma Corporation
IX51 Stereomicroscope	Olympus USA
Stereo fluorescence microscope SZX16ie204e	Olympus USA
Electronic analytical balance	Mettler Toledo Instrument Co., Ltd
Eq2200DV numerical control ultrasonic cleaner	Kunshan Ultrasonic Instrument Co., Ltd
Hpg280BX light incubator	Harbin Donglian Electronic Technology Development Co., Ltd
Spx-280B-G Boxun light incubator	Shanghai Jixing Biotechnology Co., Ltd
Eppendorf 5804R low temperature	Centrifugeg

Bench Centrifuge	
XW-80A micro vortex mixer	Shanghai Huxi analyzer Factory Co., Ltd
HL398S ultra low temperature refrigerator	Zhongke Meiling Low Temperature Technology Co., Ltd
TD5A-WS bench type low speed centrifuge	Changsha Xiangyi Centrifuge Instrument Co., Ltd

The experimental animal used in this study was zebrafish, which was provided by the Key Laboratory of drug screening technology, Institute of biology, Shandong Academy of Sciences. The feeding conditions of zebrafish are according to the OECD manual, according to the alternating cycle of darkness for 10 hours and light for 14 hours, the temperature of zebrafish breeding room (Fig. 1, 2) is controlled at 28 ± 0.5 ℃, and the bait and harvest shrimp are fed on time every day. When zebrafish embryos need to be used, we need to select healthy and mature male and female zebrafish the night before, put them into the spawning tank according to the male female ratio = 1:1 or 2:1, separate the male and female zebrafish with a partition (Fig. 3 4), remove the partition at 8:30 the next morning, and obtain embryos at 10-11. Wash the embryos with clean fish water for 2-3 times, add methylene blue for disinfection, and put the collected embryos into a 28 ℃ constant temperature incubator for standby.

Fig. 1 - Zebrafish culture room.

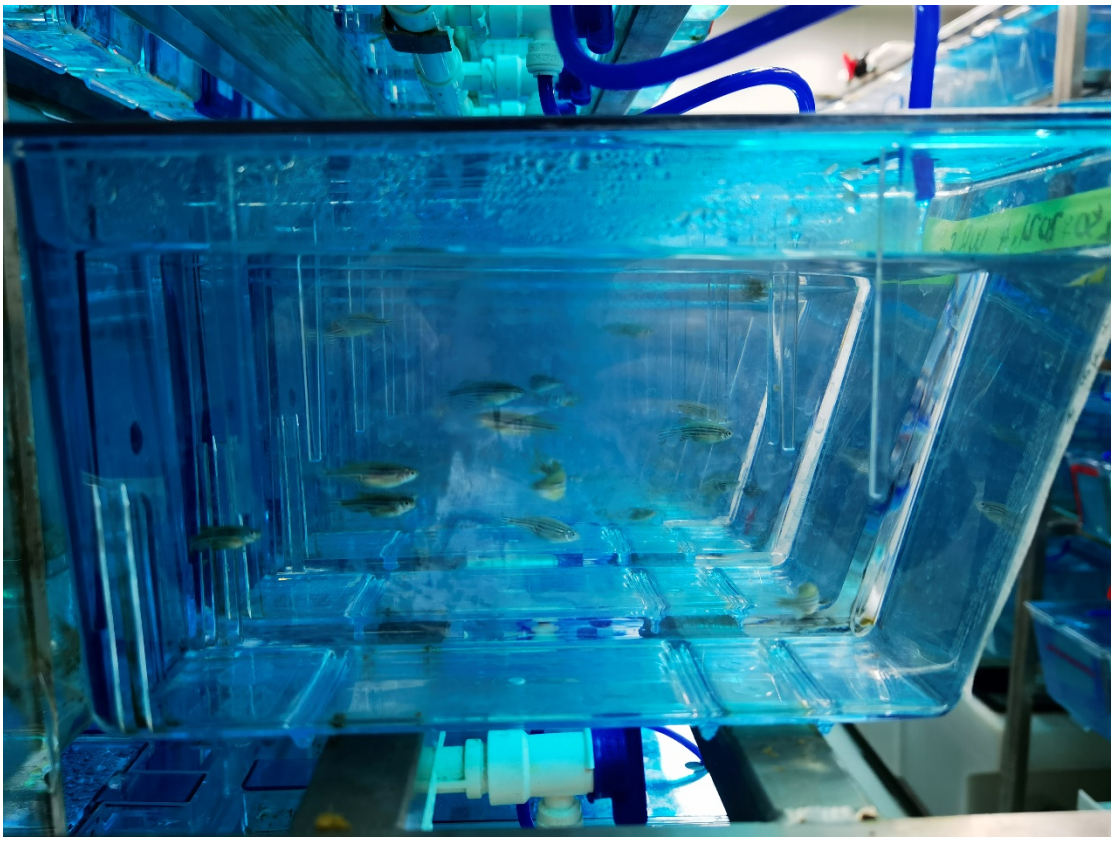


Fig. 2 - Zebrafish culture tank.

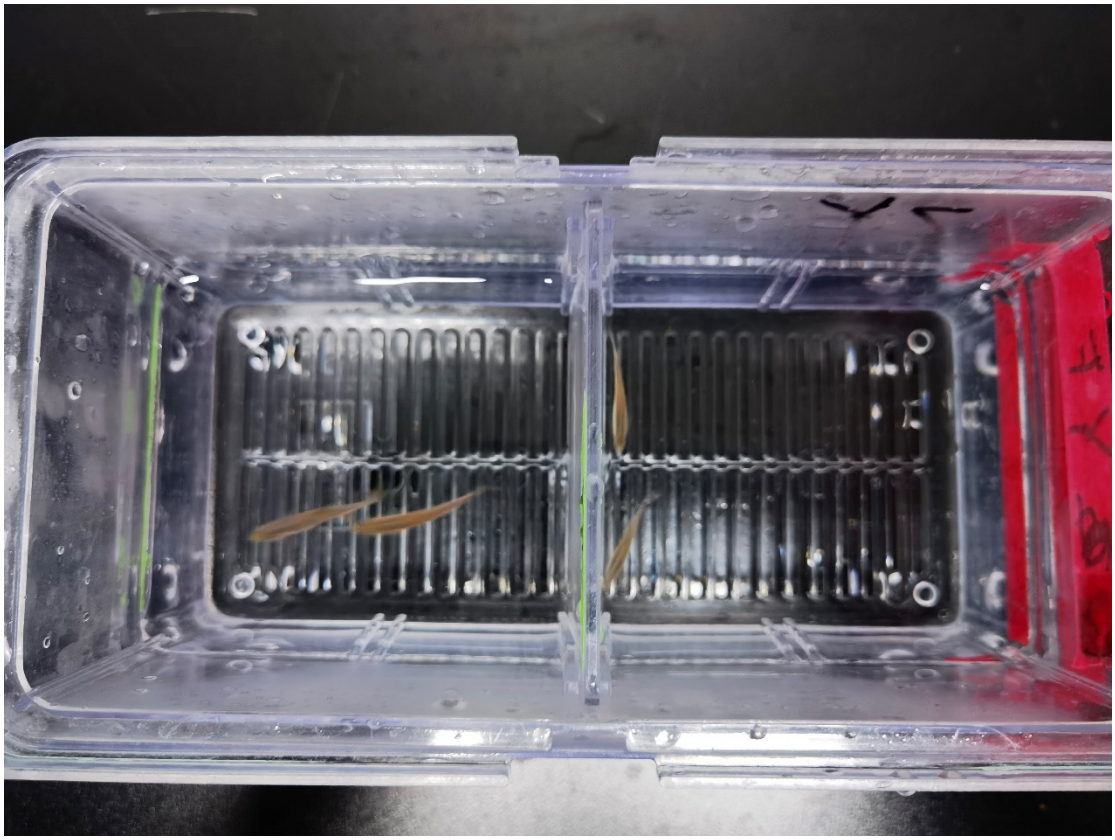


Fig. 3 - Zebrafish spawning tank.

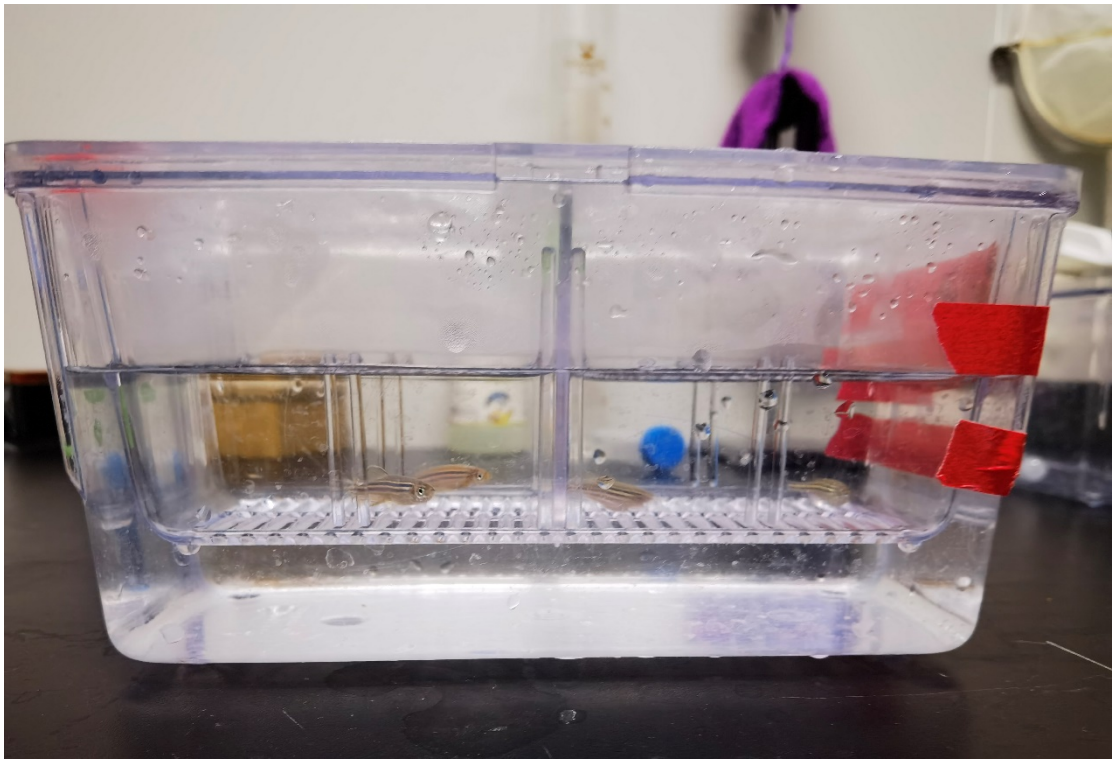


Fig. 4 - Zebrafish spawning tank.

Safety Assessment of *American ginseng* led in an experimental way. After egg collection, PTU was added to inhibit melanin production. When zebrafish developed to 3dpf, healthy young fish were selected and carefully inhaled into each hole of 6-well plate, and about 20 young fish were put into each hole. The blank control group (water prepared by the breeding system) was set, and the Administration Concentration of AGE was set to 15, 30, 45, 60 and 90 μg/mL。Each concentration group is provided with 2 multiple holes. Light controlled culture (14 / 10h, light / dark) was carried out in a constant temperature incubator at 28 ℃. After continuous administration for 3 days, the dead young fish were removed regularly every day (whether there was a heartbeat as the standard to judge whether zebrafish survived) and the liquid medicine and fish culture water of the control group were replaced. Three days after administration, the anesthetized young zebrafish were fixed on the slide in a lateral position with methylcellulose, and the morphology of zebrafish was observed (Fig. 5).

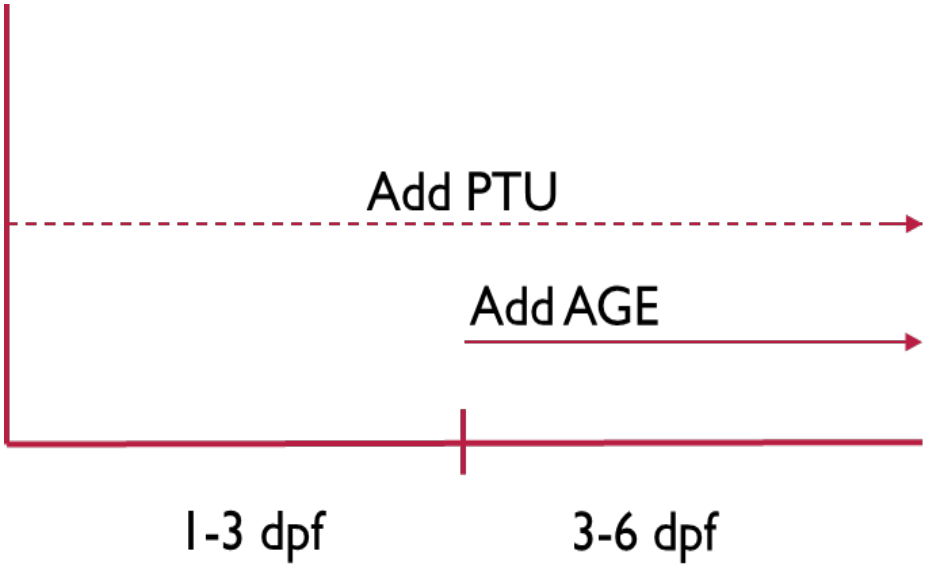


Fig. 5 - Experimental flow chart.

The zebrafish phenotype was as follows. At 3 dpf, the phenotypic changes of young fish were observed by pose microscope, including yolk sac delay, pericardial edema, pericardial hemorrhage, spinal curvature, swim bladder development and so on. Antiphlogistic effect of *American ginseng* on the LPS-induced inflammatory pattern in zebrafish evaluated experimentally. Zebrafish LPS inflammation model is a systemic inflammation model induced by LPS. LPS is the main structural component of the cell wall of Gram-negative bacteria. It is an endotoxin. It is one of the main ligands recognized by the innate immune system through pattern recognition receptors. It can cause inflammatory cascade reaction 136. It is a common inducer of mammalian inflammatory model and is also widely used in zebrafish inflammatory modeling. LPS immersion administration or yolk sac microinjection administration of zebrafish embryos 2 ~ 3 days after fertilization can induce an increase in the number of zebrafish immune cells and induce inflammatory response [137]. Whether drug treatment can reverse the increase of immune cells caused by LPS has become one of the important indexes for the evaluation of its antiphlogisticactivity.LPS was selected as the model drug to cause systemic infectious inflammation in zebrafish. Under stereomicroscope, 3 dpf Tg (zlyz: EGFP) transgenic zebrafish with normal development were selected and carefully moved into 6-well plates, with 20 young fish per well. The control group (embryo culture water), LPS (100 μg/mL) group and AGE (20μg/mL) group, LPS +10 μg/mL AGE group, LPS + 15 μg/mL AGE group, LPS + 20 μg / mL AGE group, each concentration group was set with 2 multiple wells. Light controlled culture (14 / 10h, light / dark) was carried out in a 28 ℃ constant temperature

incubator. The solution was changed every day after continuous administration for 3 days. After 72 hours of application, the aggregation of inflammatory cells of zebrafish in each group was observed under fluorescence microscope, and the fluorescence intensity was calculated (Fig. 6).

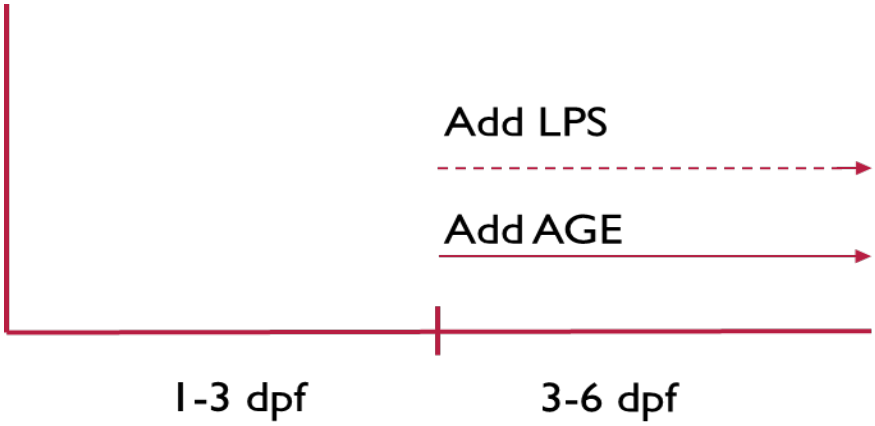


Fig. 6 - Experimental flow chart

We analyzed the received data and we can say that, the fluorescence intensity from cloaca to tail in different groups was calculated. The experimental data were expressed by mean ± SE, and the statistical difference was analyzed by ANOVA. $P < 0.05$ is a significant difference, $P < 0.01$ is a very significant difference.

The anti-inflammatory effect of American ginseng in a zebrafish model of tail-cut inflammation was evaluated experimentally. Zebrafish tail amputation inflammation model is a traumatic inflammation model. The treatment of zebrafish tail amputation can induce local damage to zebrafish tail and promote immune response of zebrafish immune cells. In the early stage of zebrafish embryonic development, macrophages and neutrophils participate in the inflammatory response together. The number of macrophages and neutrophils in the wound reaches the peak 6 hours after zebrafish tail amputation, and the inflammatory response begins

to subside 6 hours later [138, 139-142]. Researchers can study the inflammatory mechanism and evaluate the antiphlogisticactivity of drugs by describing the migration, aggregation and regeneration of immune cells after injury.

The normal 72 hpf Tg (zlyz: EGFP) transgenic zebrafish were selected under the stereomicroscope, and the tail was cut at the same position of the zebrafish tail with a lancet. The operation was carried out under the microscope. Carefully move the treated zebrafish into a 6-hole plate, 20 per hole. The blank control group (non-amputated tail treatment + embryo culture water), amputated tail group (embryo culture water) and amputated tail + 10 μg/mL age group, broken tail + 15 μg/mL age group, broken tail + 20 μg/mL age group, each group is provided with 3 multiple holes. Light controlled culture (14 / 10h, light / dark) was carried out in a 28 ℃ constant temperature incubator. After 12h of incubation, photos were taken under a fluorescence microscope to observe the migration and aggregation of inflammatory cells at the broken tail of each group (Fig. 7).

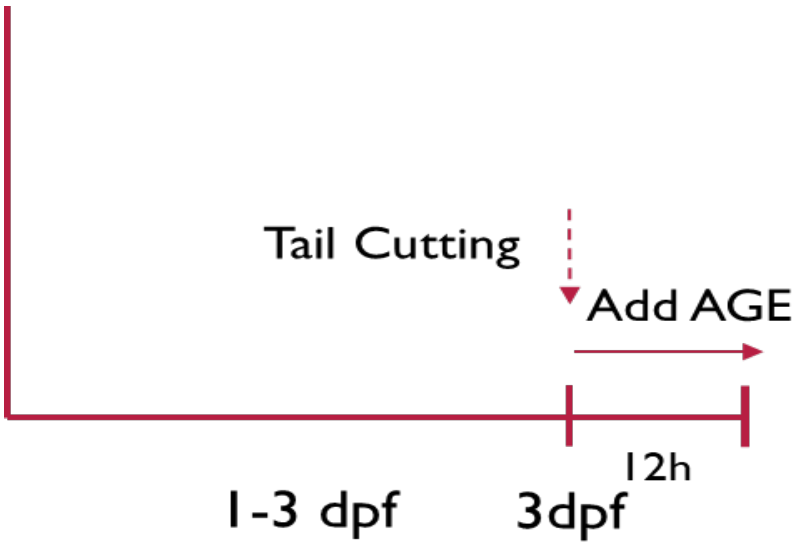


Fig. 7 - Experimental flow chart.

We have analyzed the received data and can note that, the fluorescence intensity at the broken tail of different groups was calculated. The experimental data were expressed by mean ± SE, and the statistical difference was analyzed by ANOVA. $P < 0.05$ is a significant difference, $P < 0.01$ is a very significant difference.

The anti-inflammatory activity of American ginseng was evaluated experimentally in a TNBS-induced model of intestinal microorganisms dependent on IBD in zebrafishTNBS was selected as a model drug to cause inflammatory bowel disease like the pathological manifestations of IBD patients. TNBS induction has the advantages of short modeling time, high repeatability and easy induction. It is a classic IBD chemical mutagen [143]. TNBS can cause disappearance of intestinal peristalsis, intestinal dilatation, and formation of intestinal obstruction in zebrafish. At the same time, it can also shorten the length of intestinal villi and increase the number of goblet cells, which is very similar to the pathological manifestations of IBD patients [144].

Anti-inflammatory activity was determined based on the migration of inflammatory bowel cells. Under stereomicroscope, 3 dpf Tg (zlyz: EGFP) transgenic zebrafish with normal development were selected and carefully moved into 6-well plates, with 20 young fish per well. The control group (embryo culture water), TNBS (75 μg/mL) group and AGE (20μg/mL), TNBS + 10 μg/ml age group, TNBS + 15 μg/mL age group, TNBS + 20 μg/mL age group, each concentration group was set with two double holes, and the light controlled culture (14 / 10h, light / dark) was carried out in a 28 ℃ constant temperature incubator. In the first three

days, except the control group, only TNBS was added for modeling, and the solution was changed every day. On the fourth day, wash the solution, TNBS + 10 μg/mL AGE group, TNBS + 15 μg/mL AGE group, TNBS + 20 μg/mL AGE group was added with the corresponding concentration of age for protection, and TNBS (75 μg/mL) group was added with embryo culture water. After 24 hours of protection, the migration and aggregation of intestinal inflammatory cells in each group were observed under fluorescence microscope (Fig. 8).

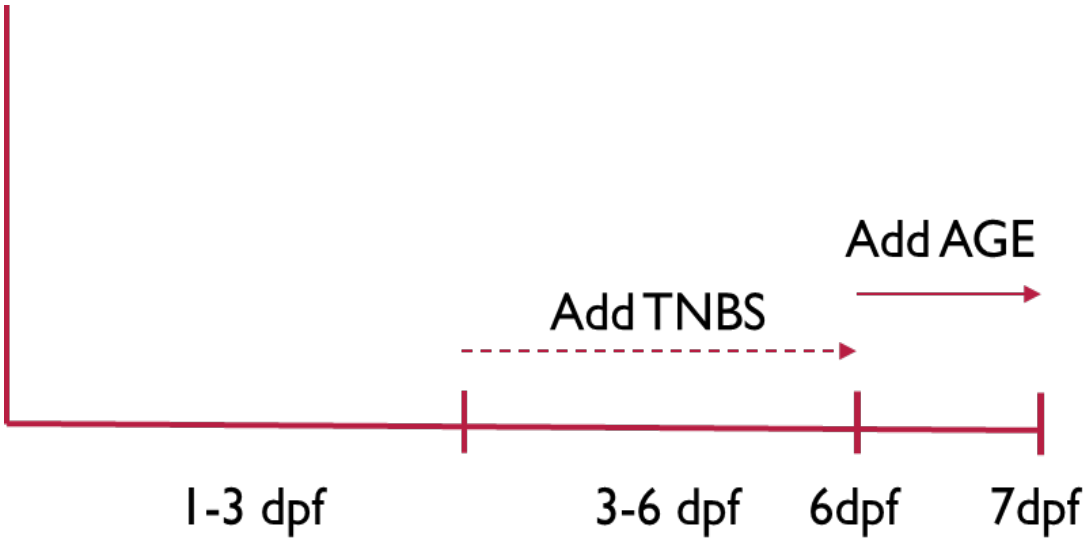


Fig. 8 - Experimental flow chart.

Determination of anti-inflammatory activity was carried out on the basis of intestinal motility. Fluorescent transgenic zebrafish with 3 dpf immune cells were randomly divided into blank control group and treatment group. The blank control group did not add any drugs and substances to be tested, and the final concentration in the treatment group was 50 μg/mL trinitrobenzene sulfonic acid. The zebrafish in each group were placed in a constant temperature incubator at 28.5 ℃ and incubated in dark for 3 days. After that, the larvae of each group were washed and placed in the calcein dye solution with a mass concentration of 0.2%, pre dyed for 1.5 h under the

condition of avoiding light, then the pictures of intestinal parts showing green fluorescence were taken under the fluorescence microscope, and the mean iod0 and iod1 of zebrafish intestines in the blank control group and the treatment group were calculated by imagepro plus software. The zebrafish in the above pre dyed treatment group were randomly divided into model group and sample group with different concentrations, with 10 larvae in each group, and two double holes were set at the same time. The larvae of blank control group and model group were placed in fresh culture water respectively, and the larvae of sample group with different concentrations were placed in culture water containing samples to be tested. The larvae of the above three groups were incubated in a constant temperature incubator at 28.5 ℃ for 16 hours without light (Fig. 10). After that, take pictures of intestinal parts showing green fluorescence under the same exposure conditions with a fluorescence microscope, and use imagepro-plus software to calculate the intestinal IOD2 of each larva in the blank control group, model group and sample groups with different concentrations. According to the formula: intestinal IOD coefficient of the blank control group = (IOD0 mean - IOD2) / iod0 mean, Intestinal IOD coefficient of model group / sample groups with different concentrations = (IOD1 mean - IOD2) / IOD1 mean, calculate the intestinal IOD coefficient of zebrafish in each group, and use GraphPad software to statistically analyze the data (Fig. 9).

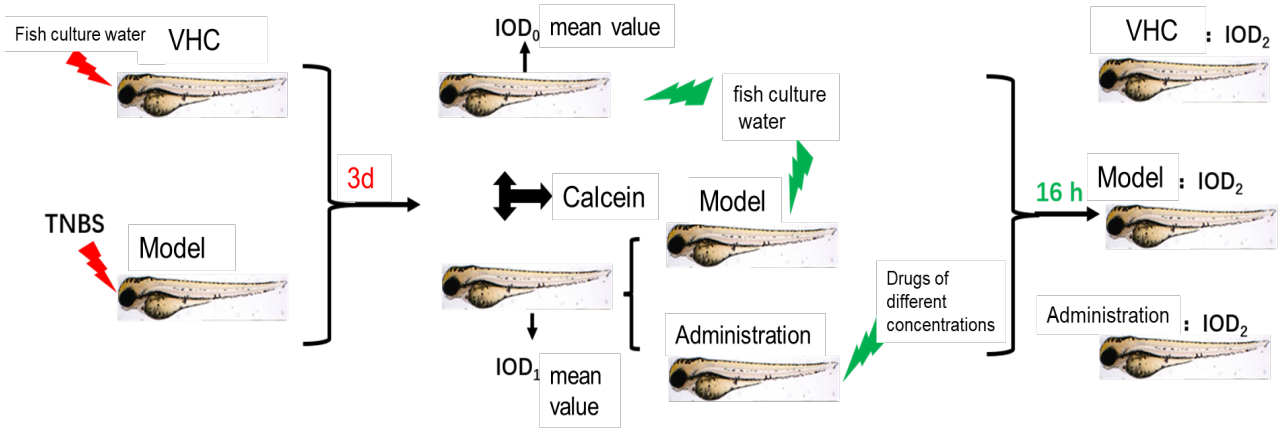


Fig. 9 - IOD calculation diagram.

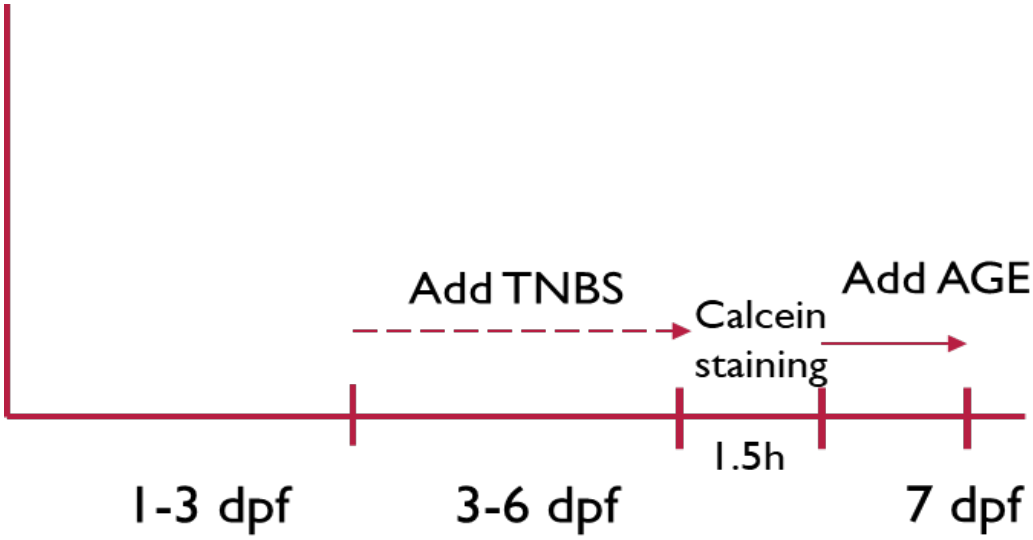


Fig. 10 - Experimental flow chart.

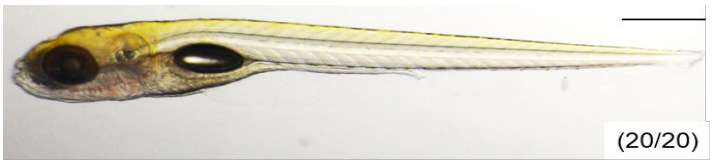
The migration number of intestinal immune cells and intestinal IOD coefficient in different groups were calculated. The experimental data were expressed by mean ± SE, and the statistical difference was analyzed by ANOVA. $P < 0.05$ is a significant difference, $P < 0.01$ is a very significant difference.

2.1. Results

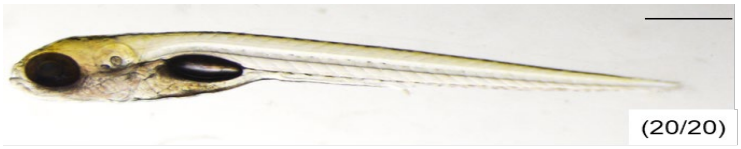
Safety evaluation of *American ginseng*. Compared with the blank control group, the low concentration AGE group 15 μg/mL, 30 μg/mL, 45 μg/mL showed no

abnormal and developmental toxicity compared with the control group. In the 60 μg/ mL AGE group, the development of swim bladder was slow (green arrow), and in the 90 μg/ mL AGE group, the development of swim bladder was slow (green arrow), and the absorption of yolk sac was delayed (red arrow). In terms of drug death, two fish died in 90 μg/mL group. According to the experimental results, when the concentration is less than 45 μg/mL, American ginseng has no toxicity and is a safe dose. At high doses of 60 μg/ml to 90 μg/ml, mild developmental toxicity was observed, but not lethal.

VHC

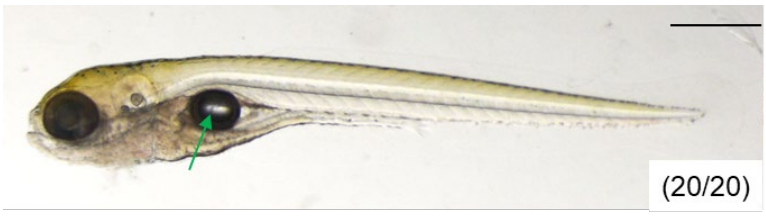


15 μg/mL

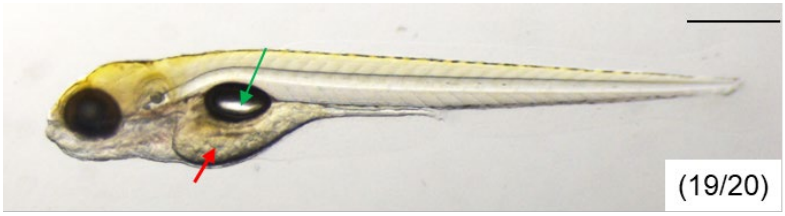


30 μg/mL

45 μg/mL



60 μg/mL

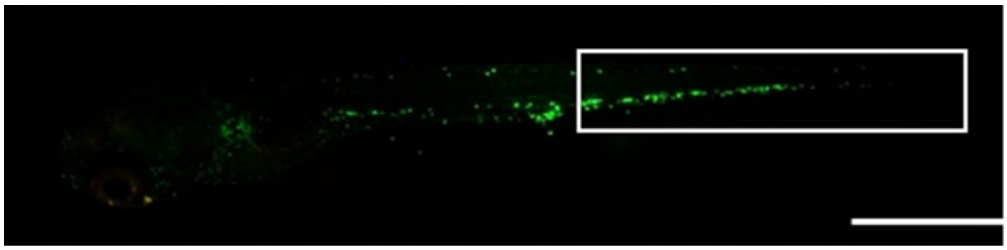


90 μg/mL

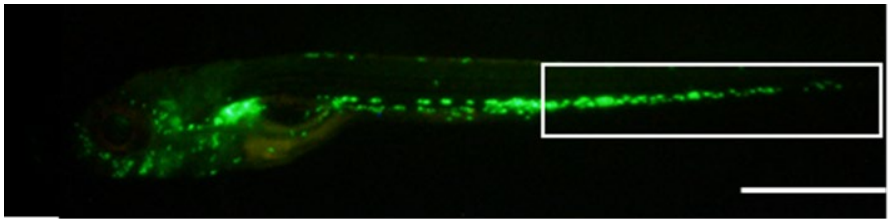
Fig. 11- Morphology of zebrafish.

Antiphlogistic effect of American ginseng on LPS-induced inflammation model of zebrafish. According to Figure 12, compared with the blank control group, 100 ug/mL LPS significantly increased the number of inflammatory cells in the body of zebrafish. Compared with LPS (100 ug/mL) model group, the number of inflammatory cells in zebrafish body in 10, 15 and 20 ug/mL AGE groups was significantly reduced, and there was a dose dependence. Image-pro was used to calculate the fluorescence intensity from cloaca to tail of each group, and Anova was used to analyze the statistical difference. It was found that there were extremely significant differences between model group and blank control group, and LPS could cause systemic infectious inflammation of zebrafish. There was no significant difference between the 20 ug/mL group and the blank control group, indicating that AGE itself did not cause the reduction of inflammatory cells. There were significant differences between 10 ug/mL group and model group, and extremely significant differences between 15 and 20 ug/mL group and model group, indicating that AGE

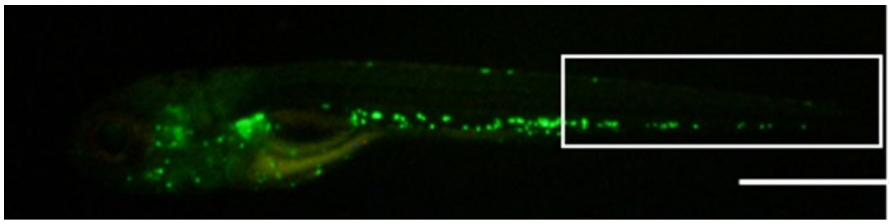
can alleviate systemic infectious inflammation caused by LPS in zebrafish, and the antiphlogisticeffect increases with the increase of AGE concentration within the safe dose.



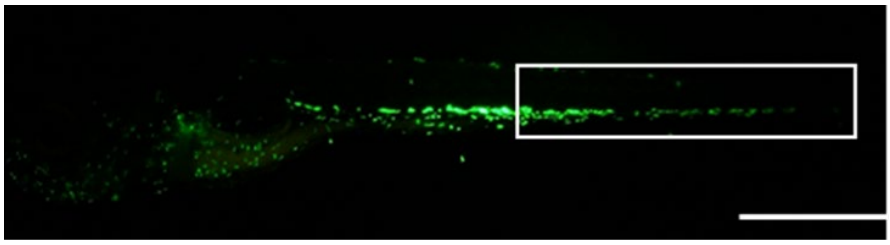
VHC



LPS 100 μg/mL



AGE 20 μg/mL



LPS 100 μg/mL+ AGE 10 μg/mL

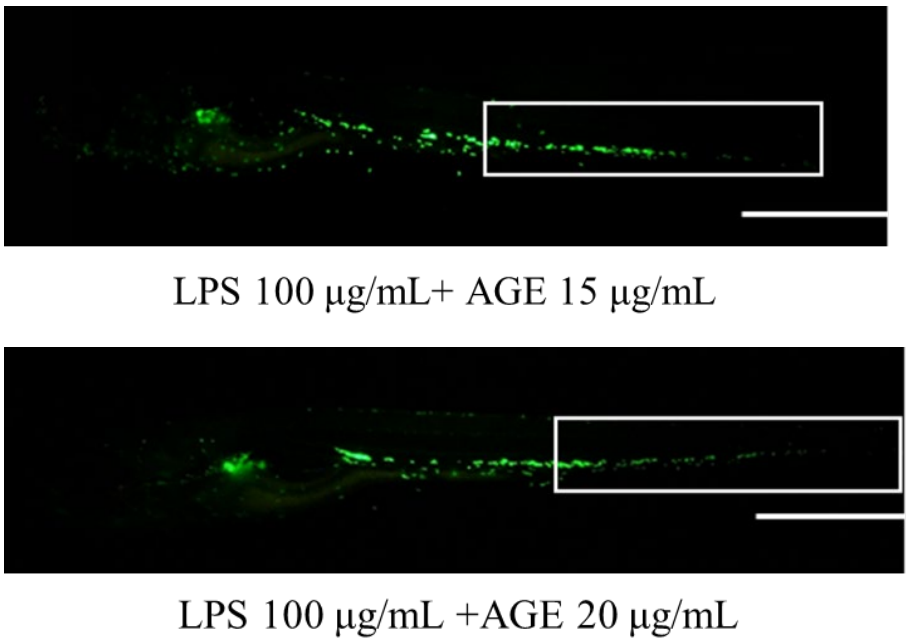


Fig. 12 - AGE inhibited LPS-induced inflammatory response in zebrafish

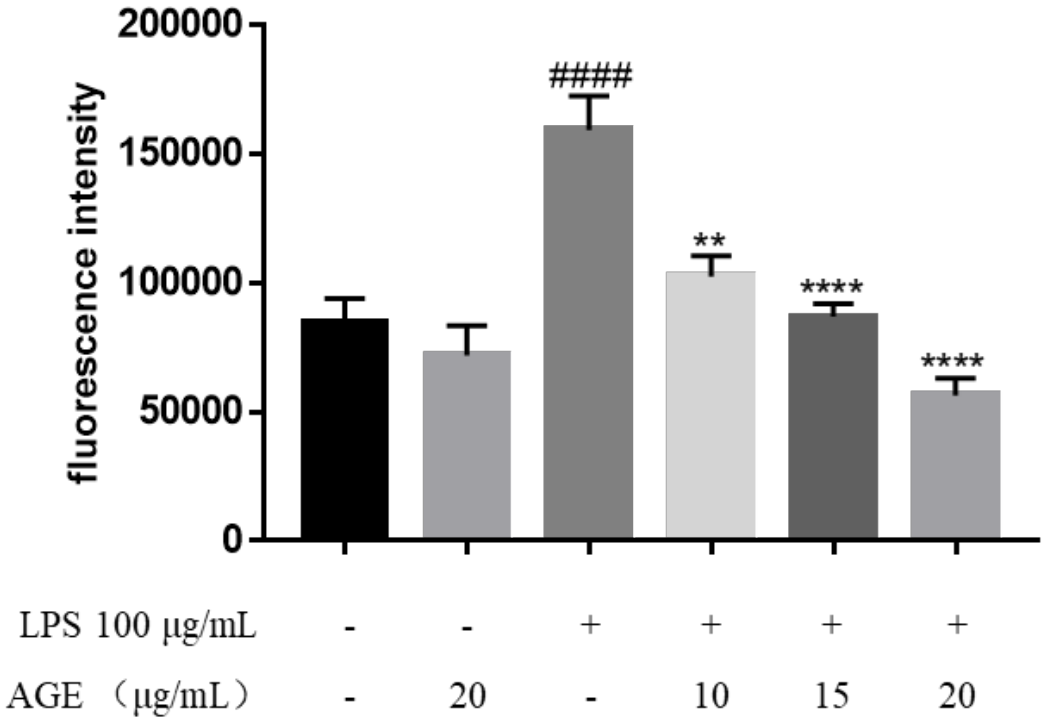
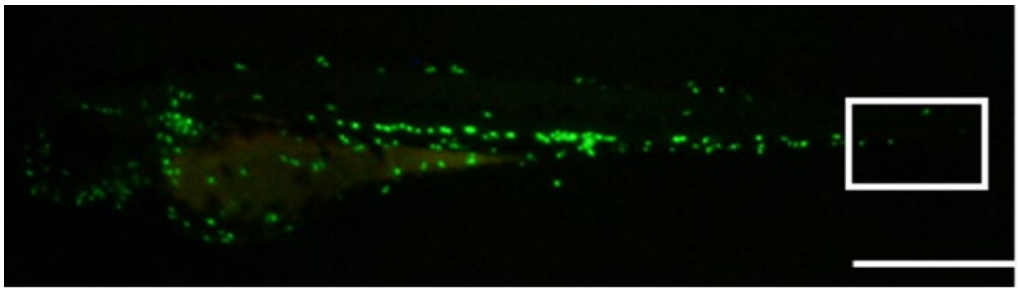
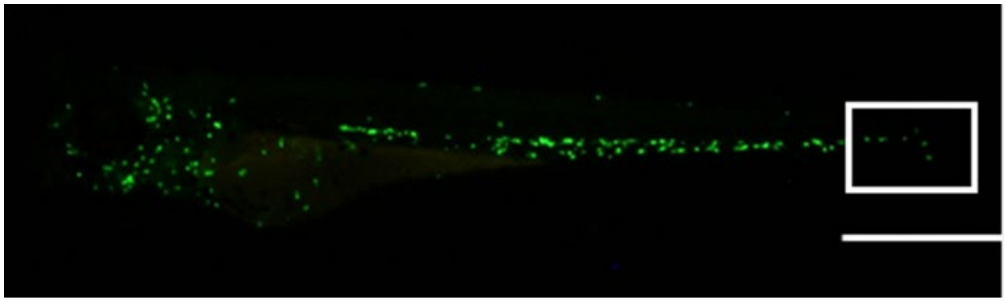


Fig. 12 - Statistics of inflammatory fluorescence intensity in the inflammatory site (white box) #p< 0.05 compared with control group, *P < 0.05, **P < 0.01, **** P< 0.0001 compared to the LPS group.

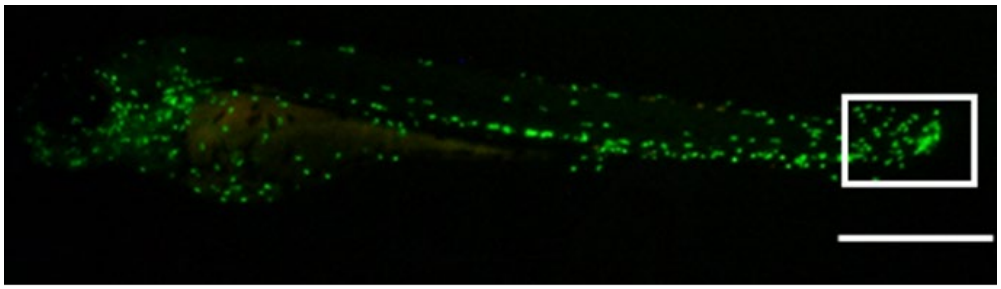
Antiphlogisticeffect of *American gin*seng on tail cutting-induced inflammation model of zebrafish. According to Figure 13, compared with the blank control group, the juvenile fishes in the tail-cutting group had obvious aggregation of inflammatory cells labeled with green fluorescence at the tail-cutting position. Compared with tail cutting model group, the number of inflammatory cells at tail severing of zebrafish in 10, 15 and 20 ug/mL AGE groups was significantly reduced, and it was dose dependent. Image-pro was used to calculate the fluorescence intensity at tail breaking in each group. Anova was used to analyze the statistical difference, and it was found that there was a very significant difference between the model group and the blank control group. It can be concluded that tail breaking can cause local mechanical injury and inflammation to zebrafish. There was no significant difference between the 20 ug/mL group and the blank control group, indicating that AGE itself did not cause the reduction of inflammatory cells. The difference of 15 and 20 ug/mL was extremely significant compared with the model group, indicating that AGE could alleviate the local mechanical injury inflammation caused by tail loss in zebrafish, and the antiphlogisticeffect increased with the increase of AGE concentration within the safe dose.



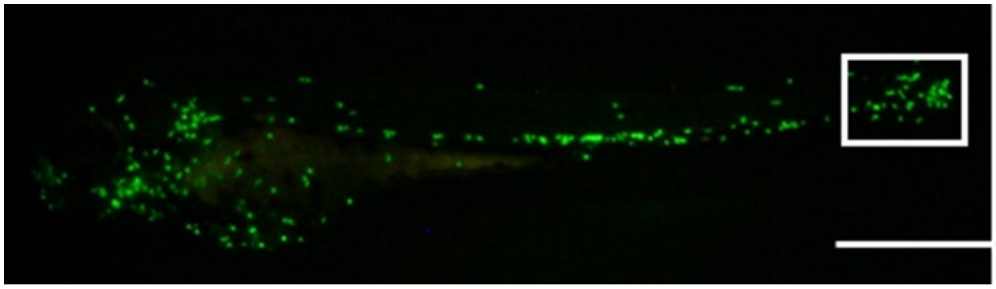
VHC



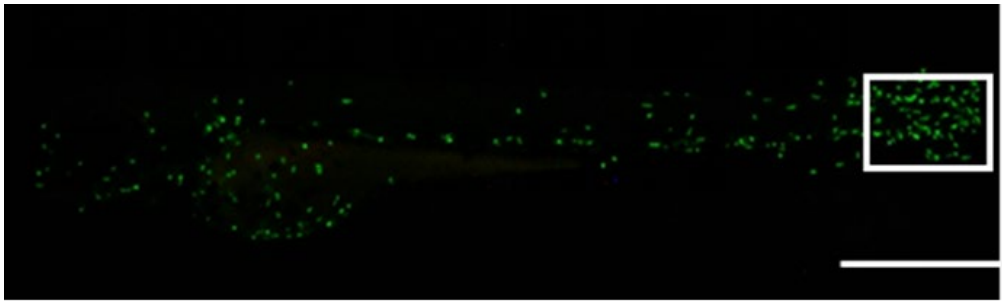
AGE 20 μg/mL



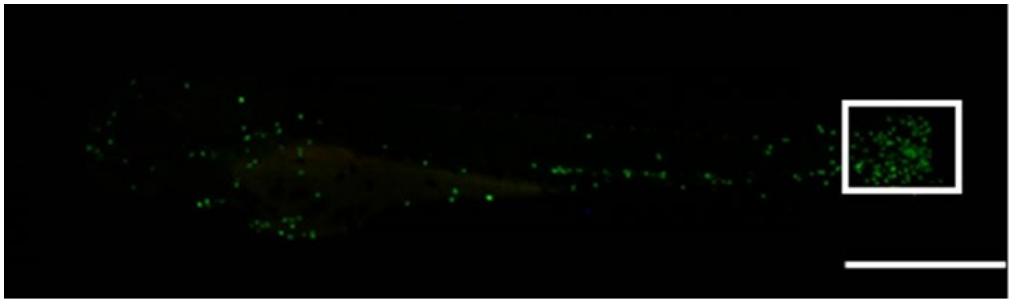
tail cutting



tail cutting+ AGE 10 μg/mL



tail cutting+ AGE 15 μg/mL



tail cutting+ AGE 20 μg/mL

Fig. 14 - AGE inhibited tail cutting-induced inflammatory response in zebrafish

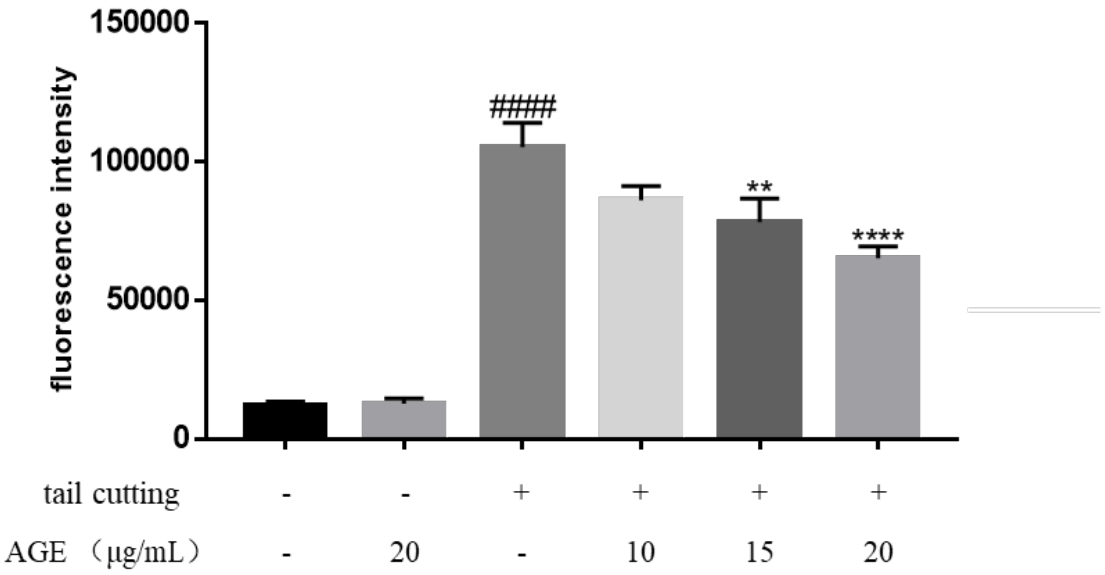
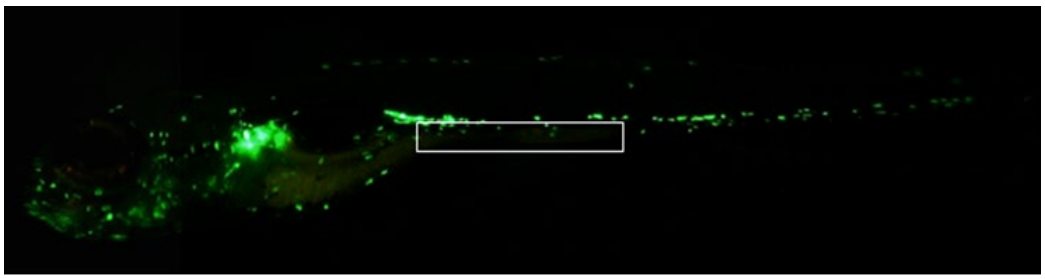


Fig. 15 - Statistics of inflammatory fluorescence intensity in the tail (white box) region, ####P< 0.0001 compared with the control group, **P< 0.01, ****P< 0.0001 compared to the tail cutting group.

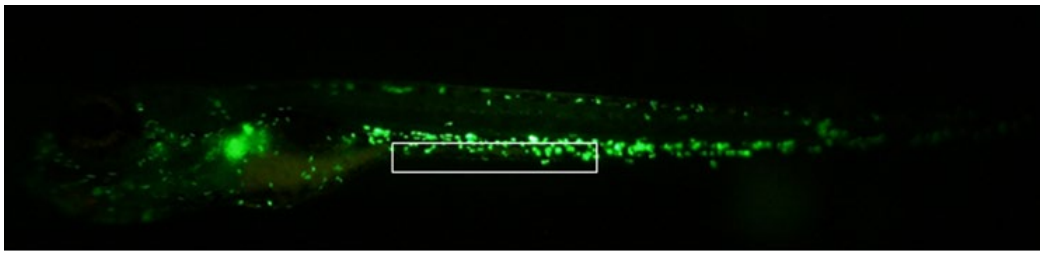
To evaluate the antiphlogisticactivity of *American ginseng* against TNBS-induced intestinal microbial-dependent IBD zebrafish model. Detection of antiphlogisticactivity based on intestinal inflammatory cell migration

According to Figure 16, compared with the blank control group, the migration number of green fluorescents labeled inflammatory cells in the intestinal position of

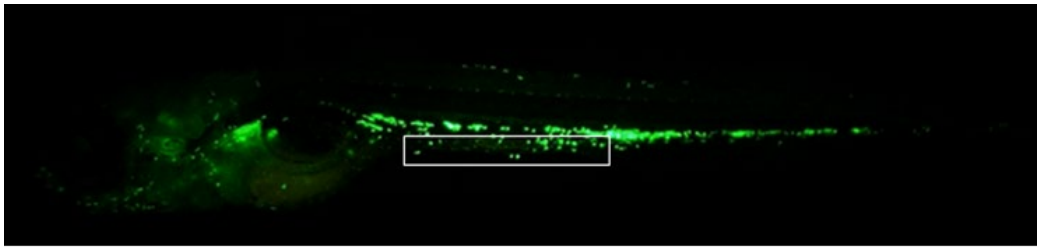
juvenile fish in the TNBS model group was significantly increased. Compared with TNBS model group, the number of intestinal inflammatory cell migration of zebrafish in 10, 15 and 20 ug/mL AGE groups was significantly reduced, with a dose dependence. The migration number of intestinal inflammatory cells in each group was counted by image-Pro, and the statistical difference analysis of ANOVA showed that there was a very significant difference between the model group and the blank control group, indicating that TNBS can cause intestinal inflammation in zebrafish. Compared with the model group, 10, 15 and 20 ug/mL showed extremely significant differences, indicating that AGE can alleviate intestinal inflammation caused by TNBS in zebrafish, and the antiphlogisticeffect increases with the increase of AGE concentration within a safe dose.



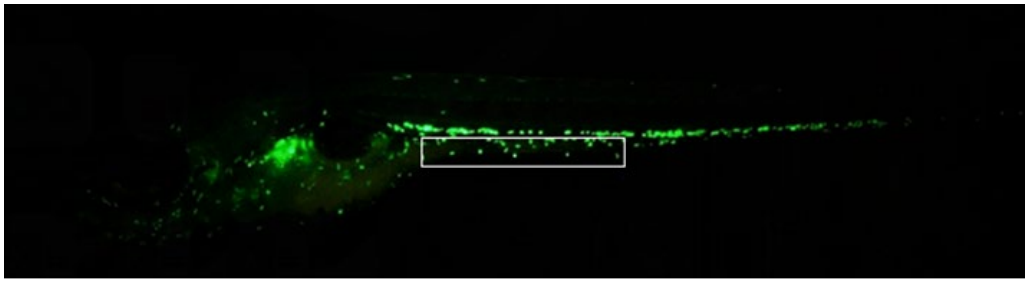
VHC



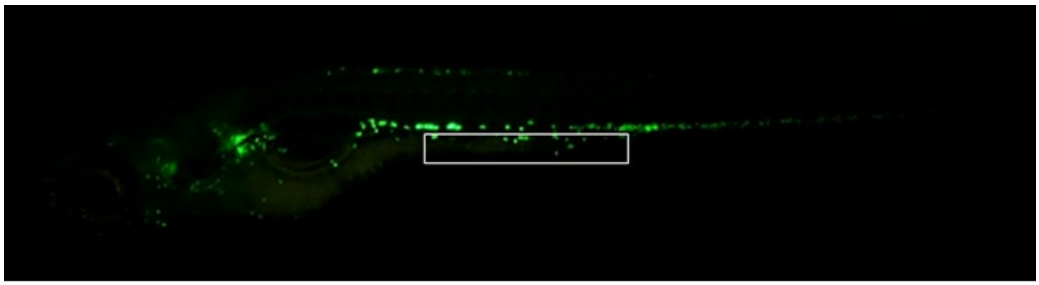
TNBS 75 μg/mL



TNBS 75 μg/mL+ AGE 10 μg/mL



TNBS 75 μg/mL+ AGE 15 μg/mL



TNBS 75 μg/mL+ AGE 20 μg/mL

Fig. 16 - AGE inhibits TNBS-induced intestinal inflammatory cell migration in zebrafish

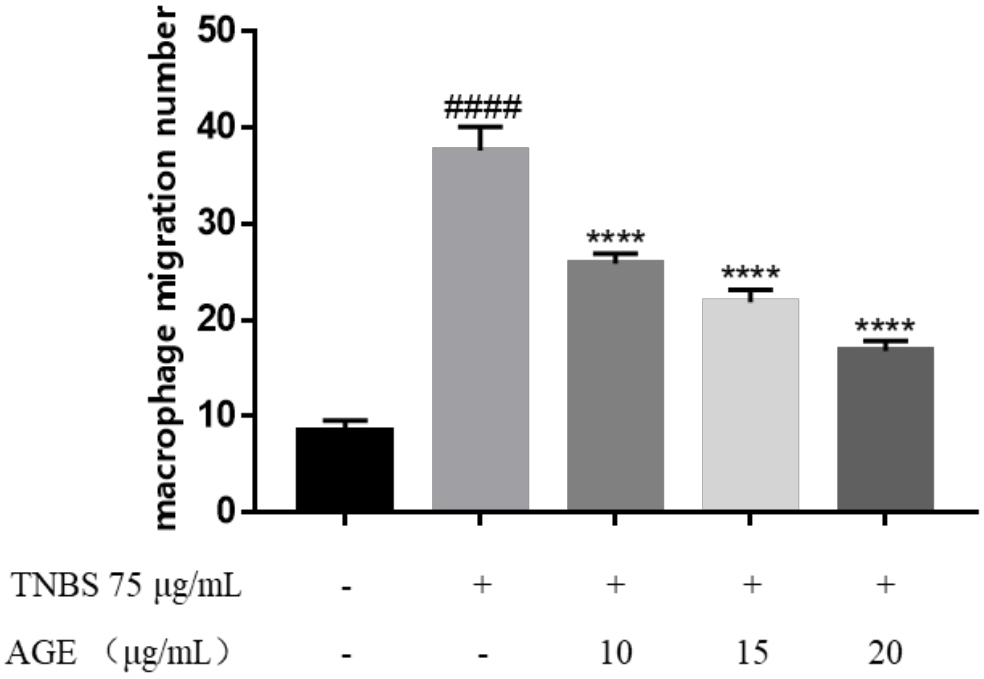


Fig. 17 - Quantitative analysis of the number of inflammatory cell migration in the same area of the intestine (white box). ####P <0.0001 compared with the control group, ****P < 0.0001 compared with TNBS model.

Detection of antiphlogisticactivity based on intestinal peristalsis. As shown in the figure, compared with the blank control group, the difference of fluorescence intensity in the intestinal tract of the blank group was significantly higher than that of the model group. However, when AGE of different concentrations was added, it could be observed that the fluorescence intensity in the intestinal tract decreased with the increase of AGE concentration. The intensity of fluorescence intensity represents the residual content of fluorescent dye in the intestinal tract of zebrafish. The lower the fluorescence intensity is, the less the residual content of fluorescent dye is, indicating the stronger the intensity of intestinal peristalsis. According to Figure 19, compared with the blank control group, the intestinal IOD coefficient of zebrafish in the model group was significantly lower, with extremely significant

statistical significance. AGE 15 and 20 ug/mL groups were significantly different from model group. As the IOD coefficient represents the ability of zebrafish to excrete fluorescent dye through intestinal peristalsis, the higher the coefficient is, the less residual fluorescent dye in the intestinal tract of zebrafish is, and the stronger the ability of zebrafish to excrete fluorescent dye is. Therefore, these results suggest that zebrafish weakened intestinal peristalsis under intestinal inflammation, and its antiphlogisticability was enhanced with the increase of AGE concentration. Within a safe dose, the antiphlogisticeffect of AGE was dose dependent.

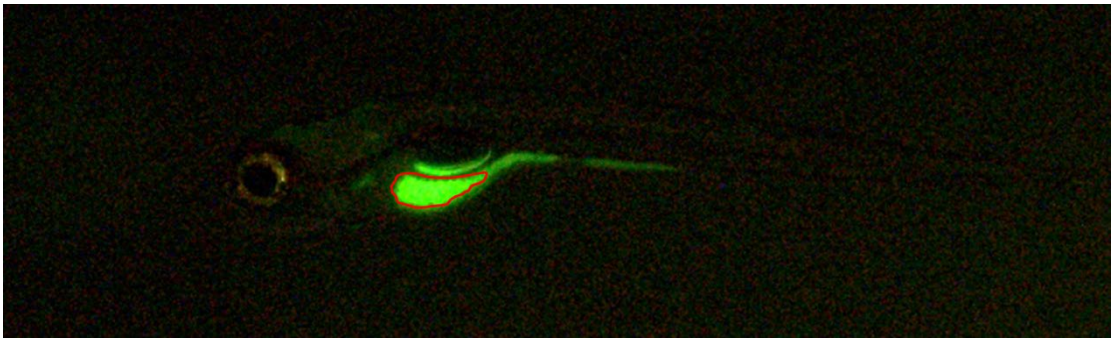
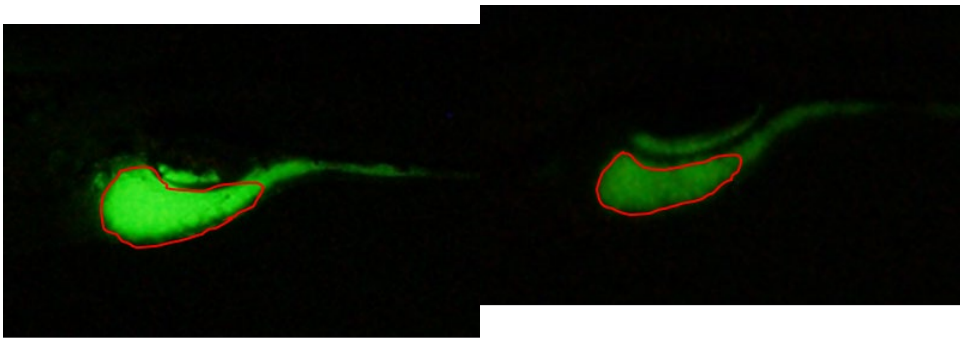


Fig. 17 - Schematic diagram of statistical area of intestinal position



VHC front VHC after

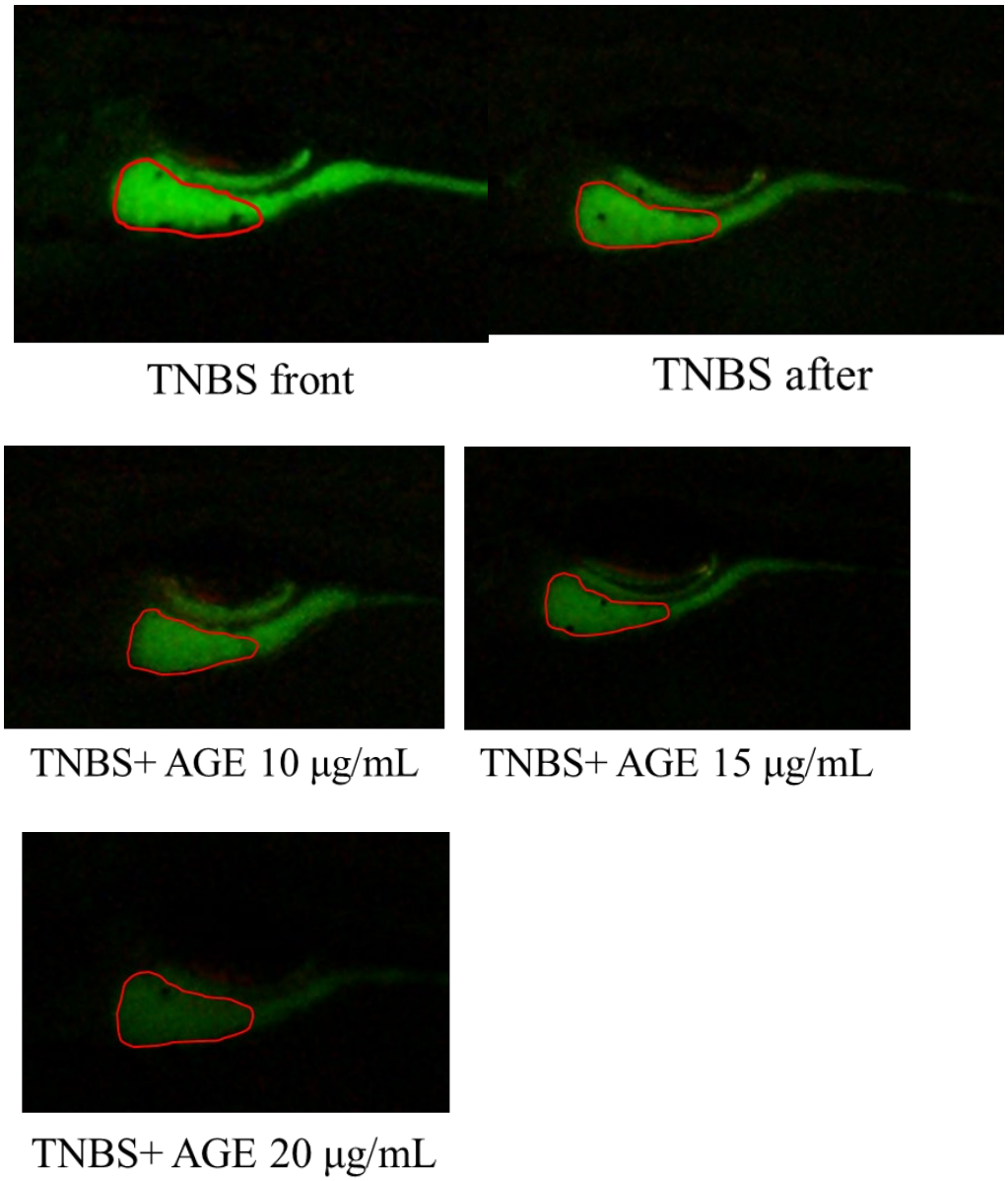


Fig. 18 - Experimental picture of intestinal peristalsis of zebrafish

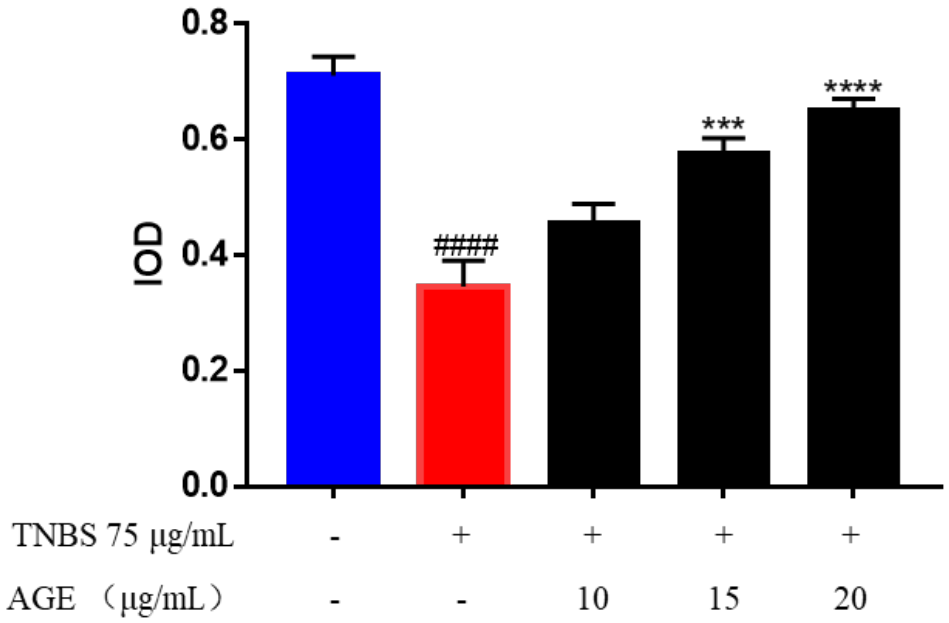


Fig. 19 - Statistical analysis of intestinal IOD coefficient. ####P < 0.0001 compared with the control group, ***P < 0.001, **** P< 0.0001 compared with TNBS model group.

2.3. Conclusion

Inflammation is one of the body's natural defense mechanisms to eliminate harmful stimuli, such as irritants or pathogens, and initiate healing. There are many causes of inflammation, such as physical, chemical, biological factors and allergy, etc. The common ones are high temperature, strong acid, bacteria and over-strong immune response caused by abnormal immune reaction state of the body, which can lead to inflammation. Inflammation can be divided into chronic inflammation and acute inflammation. Acute inflammation is a beneficial process for the body, and the occurrence of acute inflammation can help fix the injured area and accelerate the process of wound repair by the immune system [146]. Chronic inflammation, on the other hand, usually stimulates immune cells from the blood to enhance the

inflammatory response, which can destroy healthy tissue in an attempt to mistakenly start the healing process [147].

In recent years, zebrafish has been widely used in the screening of pharmacodynamic substances of TCM. As a new model building organism, zebrafish has the advantages of low cost, short life cycle, high transparency, high yield, easy maintenance and high throughput, and is an ideal vertebrate experimental model. In this study, the antiphlogistic effects of *American ginseng* were evaluated from different aspects through two inflammatory LPS, tail amputation inflammatory model and IBD inflammatory bowel disease model. Firstly, the safety of American ginseng was evaluated, and the safe concentration was selected. Subsequently, the antiphlogistic effect of American ginseng on the inflammatory zebrafish model was observed in all model groups. This study was the first to test the inflammatory bowel disease model to verify the drug activity, which provided a new idea for the follow-up antiphlogistic study of drugs, and also laid a foundation for the study of the antiphlogistic mechanism of *American ginseng*.

Section 3. Study on industrialization of sliced *American ginseng*

3.1. Advantages of traditional Chinese medicine medicinal herbs.

*American ginse*ng is a genus of *American ginseng L*. The root of the drying, native to the eastern part of North America, mainly from Canada and the United States, was introduced into China in the 17th century and has been applied for more than 300 years [148-150]. *Panax quinquefolium* has tonic properties, which can nourish qi and Yin. According to TCM theory, *American ginseng* has the tonic property of ginseng but not the dryness property of ginseng [151], and is mainly used for Yin deficiency, dryness cough, cough blood, deficiency, irritability and palpitation, insomnia, dreaminess and trance, etc. [148]. Studies on chemical composition showed that *Panax quinquefolium* contained saponins, amino acids, sugars, flavonoids and inorganic elements, among which saponins were the most important and relatively abundant active ingredients [152]. Modern pharmacological studies have shown that saponins have the effects of anti-tumor, immune regulation, improving cardiovascular diseases, affecting metabolism, anti-oxidation, antiviral, antiphlogisticand so on [153-154]. Therefore, it is more accurate to evaluate the quality of *American ginseng* with ginsenoside as the index component.

As *American ginseng* is not only a precious Chinese medicine, but also a good tonic, as a health care product of all kinds, involving pills, Powder, ointment, Dan, soup, tablet and other dosage forms, such as Panax quinquefolium buccal tablet, American ginseng royal jelly, American ginseng capsule, American ginseng tea, etc. With the increase of market demand and the extreme shortage of TCM, TCM dosage

forms (pills, powder, ointment, Dan, decoction, etc.) can no longer meet the needs of modern clinical and patients. The new dosage forms of TCM should also be developed in the direction of three effects (quick effect, high efficiency and long effect), three small effects (small size, low toxicity and low side effects) and five conveniences (easy to take, carry, produce and transport [155-156], so that the effective active ingredients in TCM can be released and utilized in an effective and maximum way.

Has several thousand years history of TCM yinpian, has experienced the development process of several generations of characteristics, with TCM yinpian ("Chinese pharmacopoeia "or local standard contains Chinese medicine yinpian), particle type yinpian (Japan or industrial feeding slices) at home and abroad, single extract granule formula particles (China) and the Chinese traditional medicine superfine yinpian (broken wall single taste traditional Chinese medicine yinpian or Taiwan powder), etc. On the premise of following the theory of TCM, "inheritance never leaves the ancient, innovation never leaves the root" is to explore the essence of TCM decoction pieces, and it is also the starting point of scientific and technological innovation of TCM decoction pieces. On the premise of retaining the whole composition of TCM pieces, the plant cell wall is broken and crushed modern ultra-fine air crushing technology. The powder of 45 um can fully release the effective components of TCM, thus greatly improving the efficacy of TCM. Then, the 30-100 mesh particles can be made by using the non-additive excipient technology [157], which is a new type of TCM decoction pieces with high utilization and convenient portability.

The key technical problems in the production of broken Chinese medicine decoction pieces are the technology of broken Chinese medicine decoction pieces ultrafine grinding technology and granulation technology of unreinforced excipient. Compared with traditional decoction pieces, ultra-micro (wall broken) decoction pieces of Chinese medicine have the following advantages :(1) the whole composition is retained, which is consistent with the material basis of the original traditional decoction pieces and retains the properties of Chinese medicine decoction pieces [157]; (2) Improve the dissolution rate and cumulative dissolution rate of active ingredients, and increase the bioavailability of drugs [158]; Improving the efficacy, reducing the dosage, and reducing the waste of TCM is a technological progress to protect the resources of TCM, which can greatly alleviate the current situation of extreme shortage of TCM resources [159]; (3) uniform quality, control, to improve the quality of the Chinese medicine yinpian, at the same time, compared with other innovation is easy to be set up based on the nature of biological or chemical composition on the basis of quality control system, also easy to establish the safety indexes based on the requirement of oral agents or green products, including microbial indicators, pesticide residues, heavy metals, aflatoxin, sulfur dioxide, etc., It can comprehensively evaluate the quality of Chinese medicine ultrafine (broken wall) decoction pieces, which is beneficial to scientific evaluation and supervision of Chinese medicine decoction pieces; (4) The application mode is simple, fast and flexible, which can meet the requirements of modern fast-paced and high-standard life [160-164].

In recent years, wall-breaking crushing technology has been widely applied in the field of TCM, and the varieties of wall-breaking decoction pieces of TCM have gradually increased. The research on the evaluation, industrialization and application of wall-breaking decoction pieces of TCM has become a popular direction of inheritance, improvement and innovation of TCM decoction pieces, and some achievements have been achieved. This topic takes *Panax quinquefolium*, which is widely used in daily health care, as the research object, and studies the possibility of the application of wall breaking technology of TCM in *Panax quinquefolium*, to provide strong theoretical basis and support for industrial production research.

Increase the dissolution of active ingredients, improve the extraction rate of active ingredients, enhance the efficacy of drugs. The therapeutic effect of TCM decoction pieces is mainly based on the active ingredients in the drugs, most of the active ingredients in the cells, so the dissolution rate of the active ingredients is low. Wall breaking decoction pieces of traditional Chinese medicine use modern ultra-fine grinding technology to crush traditional Chinese medicine with cell structure to D90 < 45μm Homogeneous powder less than m (more than 300 mesh), because the cell wall breaking rate of medicinal materials can reach more than 95%, compared with the traditional decoction pieces, it is more conducive to the release of active components in cells, thus greatly improving the dissolution rate of active components.

Chen Xi [165]. studied the content of active components in c. deserticola before and after wall breaking, and the results showed that the content of echinoside in c.

deserticola before and after wall breaking was (4.36±1.14) mg/ ml higher than that in the normal group (3.12±0.95) mg/ ml, and the difference was statistically significant ($P < 0.01$); The content of acylate in broken group was (0.14±0.03) mg/mL higher than that in normal group (0.07±0.02) mg/ ml, and the difference was statistically significant ($P < 0.01$). Feng Hua et al. [166] measured the content of volatile oil and chlorogenic acid in the broken and non-broken samples of Lonicerae japonica before and after wall breaking and crushing, and the results showed that the content of volatile oil in the broken and non-broken samples was 0.481%, which was higher than that in the non-broken group (0.123%). The content of chlorogenic acid in the broken wall group was 5.31% higher than that in the non-broken wall group (3.24%), the difference between the two groups was statistically significant ($P < 0.01$). Xu Haoqi et al. [167] tested the ginsenoside content of traditional decoction pieces, crude powder and broken wall decoction pieces of *American ginseng* in water and simulated gastric juice respectively, and the results showed that the dissolution amount of broken wall decoction pieces was 14% and 8% higher than that of traditional decoction pieces and crude powder after 5 min in water. The dissolving amount of wall breaking powder in simulated human gastric juice was 5% and 14% higher than that of traditional decoction pieces and crude powder. Huang Yirong et al. [168] conducted a comparative study on the coagulation effect of conventional decoction pieces of Panax ginseng and wall-broken decoction pieces of Panax ginseng with rabbits, and the results showed that the coagulation time of the wall-broken group was significantly shorter than that of the conventional decoction pieces group, and the difference was statistically significant ($P < 0.05$),

the efficacy of Panax ginseng decoction pieces was significantly better than that of the conventional decoction pieces group. Huang Qichun et al. [169] measured the total flavonoids content of ginkgo biloba wall-broken decoction pieces and conventional fine powder respectively, and the results showed that the total flavonoids content of ginkgo biloba wall-broken decoction pieces was higher than that of conventional fine powder, and the difference was statistically significant ($P < 0.05$), it can be seen that wall crushing can improve the dissolution amount of total flavonoids in *Ginkgo biloba* leaves. He Yiheng et al. [170] studied the yield of astragalus polysaccharides in different particle sizes of astragalus membranaceus before and after wall breaking, and divided astragalus membranaceus into decoction piece group, 80 mesh group, 200 mesh group, 400 mesh group, 600 mesh group and 800 mesh group. Astragalus membranaceus polysaccharides were extracted by water method and CAO method at 70℃, 80℃ and 90℃, respectively. When astragalus polysaccharides were extracted at 80℃ by water method or CAO method, the yield of *Astragalus polysaccharides* in 800 groups was 8.36% and 8.49%, respectively, while the yield of astragalus polysaccharides in conventional decoction pieces was only 2.25% and 4.87%, indicating that the yield of astragalus polysaccharides after astragalus membranaceus was much higher than that in conventional decoction pieces.

It can be seen that after Chinese medicinal materials are processed into broken decoction pieces, the cell wall of the medicinal materials is broken, the particle size is reduced and the surface area is increased, which improves the dissolution of the active components and is more conducive to the absorption of

drugs in the gastrointestinal tract, thus greatly improving the release speed and absorption degree of drugs in the body and enhancing the efficacy of drugs.

Increase the utilization rate of medicinal materials, avoid waste and protect endangered medicinal materials resources. It has been reported that traditional methods are easy to cause waste of resources for medicinal materials with strong fiber, such as astragalus membranaceus. If ultra-fine wall-breaking crushing is used, the waste in all links can be reduced [171], and 30%-70% of the original medicinal materials can be saved by wall-breaking crushing. If the wall-breaking powder of medicinal materials is processed into pellet powder, The dosage can be reduced to 1/3~1/5 of the original dosage, and the decoction only needs 1/20~1/5 of the original dosage [172]. Other drug delivery methods, such as local, acupoint and transdermal delivery, can also achieve the same therapeutic effect with a smaller dose [173]. Yang Zerui [174], such as preliminary studied the different gradient of rhodiola wall-breaking yinpian dose and conventional rhodiola yinpian effect on the intestinal flora in mice, respectively by clinical commonly used doses of 1/2, 1/4, 1/8 wall-breaking yinpian and clinical commonly used doses of rhodiola rosea rhodiola conventional slices in mice, the results show that the The intestinal flora richness of 1/8 of the decoction pieces with clinically common dose of wall breaking was higher than that of the conventional decoction pieces with clinically common dose, and the difference was statistically significant ($P < 0.05$), and the diversity of intestinal flora was higher than that of other groups, and the colony formed was relatively stable. It can be seen that taking 1/8 of the clinically common dose of rhodiola wall broken decoction pieces has the effect of regulating gastrointestinal flora. Cheng Jin-le et al.

[175] conducted a control experiment in mice by intragastric administration of wall broken decoction pieces and traditional decoction pieces against ulcer, and the results showed that the incidence of ulcer in mice in the wall broken decoction pieces group was lower than that in the traditional decoction pieces group ($P < 0.05$), and the dose was smaller than that of traditional Codonopsis pilosula decoction pieces. It is reported that [176] when TCM wall-broken decoction pieces produce the same curative effect as traditional decoction pieces, the dose required by TCM wall-broken decoction pieces is only about 1/3 of that of traditional decoction pieces, saving 2/3 of the original medicinal materials and greatly alleviating the current shortage of TCM resources.

Thus, adopting physical way of broken wall pieces processing preparation of all components without adding Chinese medicine yinpian, broken wall intact properties and chemical composition of Chinese herbal medicine and Chinese medicine yinpian, and at the same time, compared with the traditional yinpian, with less medicine treatment can achieve the desired effect, improve the utilization of medicinal materials, reduce the waste of Chinese medicinal materials, It can greatly alleviate the extreme shortage of TCM resources.

Free frying, easy to store, carry and use. The wall slices fully retained the original medicinal materials of traditional Chinese medicine effective active ingredient, bubble can swallow or directly take, avoid decoction, but also to avoid the traditional decoction and the effective components of damage caused by the long Fried or loss, easy to store, carry, to meet fast-paced life of common people now demand for health care [177].

TCM decoction pieces broken by the wall retain the basic characteristics of TCM decoction pieces, such as four qi and five tastes, performance meridian, function and indications. Meanwhile, it has the characteristics of TCM decoction pieces based on syndrome differentiation and treatment, adding and reducing with the syndrome, and has the characteristics of high efficiency and free decoction, easy to carry, controllable quality, convenient sanitation, good uniformity, ensuring the clinical application efficacy. In its processing, the physical method is adopted, without the addition of all components, and does not change the properties of chemical components of Chinese medicinal materials and Chinese herbal decoction pieces. The surface area is significantly increased, and the dispersibility and solubility are better than the traditional decoction pieces, which is more conducive to the absorption and utilization of drugs in the gastrointestinal tract. In addition, the wall-breaking decoction pieces of TCM improves the utilization rate of medicinal materials, reduces the waste of TCM, and effectively protects the resources of TCM, which is one of the main directions of the modernization of TCM decoction pieces and the future development direction of TCM decoction pieces.

3.2. Preparation method of components dosage forms for traditional Chinese medicine

3.2.1 Comminution Procedure

The raw material of TCM was crushed to get more than 300 mesh wall breaking powder. If more than 300 mesh of TCM wall breaking powder can be obtained, there is no special limit on the crushing method. Among them, the first crushing, the second crushing and the third crushing for different types of crushing. Through three

different types of grinding, it is preferred to make the API go through universal grinding (Figure 1) → ball grinding (Figure 2) → air flow grinding (Figure 3), so that the yield of wall breaking grinding can be improved after three times of grinding. For example, the original medicinal materials rich in fiber are directly air flow grinding without universal grinding and ball grinding. Many fibers cannot meet the requirements of wall crushing, resulting in waste of the original medicinal materials. The yield of wall breaking powder obtained by air crushing is more than 95% after universal crushing and ball grinding of Chinese medicinal materials powder (if the previous two crushing is not performed, the yield can only be about 84% by air crushing). Airflow crushing compressed air or superheated steam through the nozzle to generate high-speed airflow, and the formation of a high velocity gradient near the nozzle, supersonic high turbulence generated through the nozzle as a particle carrier for crushing, especially suitable for the crushing of unstable substances containing heat sensitive components. It ensured the retention of alcohols and their glycosides, flavonoids, terpenoids, phenols and their glycosides, polysaccharides, coumarins and volatile oils. The first stage of grinding is optimized for grinding dry raw materials to 60 ~ 100 mesh by universal grinding, preferably 70 ~ 100 mesh. The second stage of grinding includes grinding the first stage to about 100-500 mesh, preferably 100-300 mesh through ball grinding. The third stage of crushing consists of pulverizing the second stage to more than 300 mesh, preferably 400 ~ 1200 mesh, preferably 400 ~ 800 mesh, to obtain the wall breaking powder of Traditional Chinese medicine. If it is less than 400 mesh, the inclusion effect is poor in late inclusion pelleting, and 30-200 mesh particles cannot be effectively formed.

30-100 mesh particles and 35-80 mesh particles are preferred. On the other hand, if it is larger than 800 mesh, the solubility of the obtained particles becomes poor. The first stage crushing is preferably universal crushing. In the sample implementation, universal crushing is as follows: production capacity 300-1000 kg/h, feed particle size 8-32mm, feed moisture below 9wt %, spindle exclusive 3600rpm, yield above 98%. The required particles of 60 ~ 100 mesh can be obtained under the above conditions. The second stage grinding of the invention is preferably ball grinding, which comprises a process of ball grinding the first stage grinding material into crude powder of Chinese medicinal materials. The yield of ball milling was 97% ~ 98%. The air pulverization of the invention includes putting the crude powder of Chinese medicinal materials into an air pulverizer for air pulverization and crushing it to 300 ~ 1500 mesh to obtain the wall breaking powder of Chinese medicinal materials. Specifically, the crushing conditions include material pressure 0.5 ~ 0.9 MPa, preferably 0.6 ~ 0.8 MPa, frequency 35 ~ 45Hz, lead air pressure difference 0.2 ~ 0.8 MPa, material water content below 9wt %, preferably below 7wt %. By compressed air after filtering and dry high-speed jet into the crushing chamber, in high-pressure air more than the intersection of the material being repeated collision, friction, shearing and crushing, after crushing material with the updraft movement under the action of a draught fan to grading, rotating at high speed the classification of turbine under the action of the strong centrifugal force, make the thickness of material separation, The fine particles that meet the particle size requirements are collected by cyclone separator and dust collector through the classification wheel, and the coarse particles are dropped to the grinding zone for further grinding. The

condition of freeze drying is to get brittle material because freeze drying can get Chinese medicinal materials with higher brittleness, to ensure that air comminution can crush Chinese medicinal materials more easily and get excellent wall broken pieces.

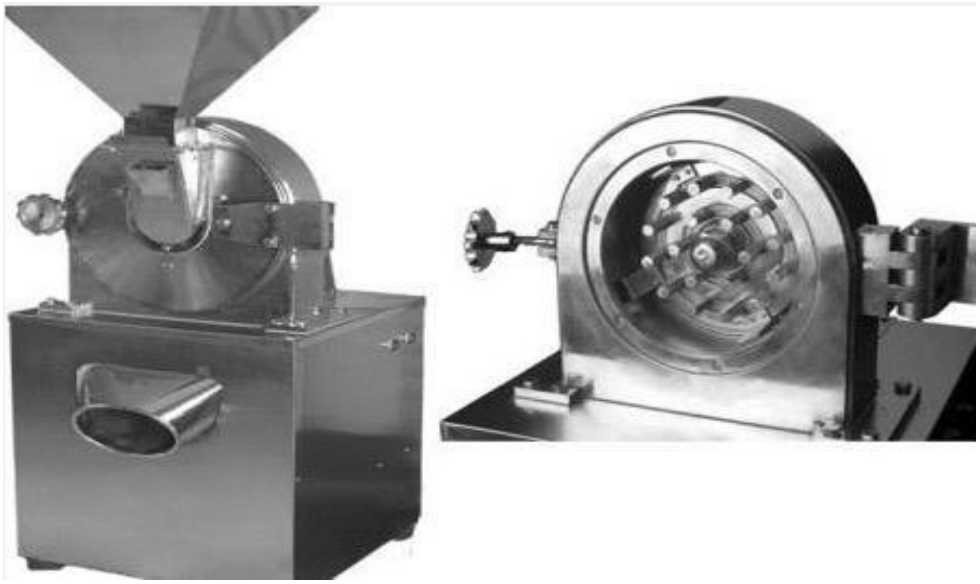


Fig. 1 - Universal Crusher.

Fig. 2 - Ball Mill.

Fig. 3 - Airflow Crusher

3.2.2. Pelleting process of micropowder

Including the traditional Chinese medicine wall breaking powder under the condition of not adding any auxiliary materials or additives for the inclusion of micro powder granulation to obtain the Angle of rerest is less than 40° and 30 ~ 200 mesh particles. In the field of TCM wall breaking decoction pieces, in order to meet the requirements of wall breaking, TCM particles need to be crushed to more than 300 mesh. At this particle size, the fluidity of decoction pieces is very poor, and it is difficult to fill in the production process and the weight difference after filling is large. When the particle size of traditional Chinese medicine particles is optimized at 400 ~ 800 mesh, large particles with excellent volume and divergence can be obtained by dry pressing, without adding any auxiliary materials or additives, such

as adhesives. The Angle of repose of the obtained particles is less than 40°, and the particle size can be controlled to 30 ~ 200 mesh. The forming pressure is generally about 70 ~ 130kg, the maximum pressure is 200 bar (1bar is equal to 1kg), the screw speed is 25 ~ 120rpm, the final speed is 8 ~ 28rpm, and the whole grain speed is 80 ~ 200rpm. Finally, 30 ~ 200 meshes of TCM decoction pieces were obtained. The Angle of repose was taken as the evaluation index to granulate micropowder without adding any flow aid. The traditional Chinese medicine powder with good wall breaking can be aggregated by physical method. In this step, the pulverized powder after breaking the wall is encapsulated in micro powder. The physical method refers to that one broken powder molecule wraps another or more broken powder molecules to form a unique molecular complex. The packaging material and core material of this complex are the same kind of medicinal material broken powder. If not treated with micro powder bag also grain method, the original broken wall powder generally greater than the Angle of 40° illiquid, after is difficult and in the process of production filling weight difference is bigger, after powder bag also grain in less than 40°, the Angle of molecular complex stability and received a significant boost liquidity, greatly improving the production efficiency, and reduce the volume difference between finished products. The advantages of the micropowder encapsulation technology of the invention include that the prepared decoction pieces have the characteristics of strong fluidity, good stability, high safety, easy dispersion and convenient administration, etc.

Drying Procedure. It is usually carried out before the comminution step, which involves freeze-drying the pretreated material to obtain the dried material. Freeze

drying can greatly improve the brittleness of raw materials of Chinese medicinal materials, which is conducive to subsequent comminution. It is mainly divided into freezing step, sublimation step and re-drying step.

Freezing Procedure. After processing of Chinese herbal medicine raw material under the atmospheric pressure precooling temperature to the first, and keep 3 ~ 4.5 hours, then cool under pressure from the first to the second temperature, and keep in 4 ~ 6 hours, optimizing 4 ~ 5.5 hours, preferred 4. 5 ~ 6 hours, after further to third temperature cooling, and keep 3 ~ 5 hours, optimizing 3. 5 ~ 4.5 hours, 4 to 4.5 hours is preferred. This freezing step is different from the traditional cooling method with linear rate, which is more conducive to the formation of specific size of ice crystals, reduces the resistance of water vapor sublimation, and makes the dry matter more brittle, which is more conducive to the subsequent grinding steps. Preferably, the first temperature is 1.5 ~ 3℃, the second temperature is -45℃ ~ -35℃, and the third temperature is below -65℃. Preferably, the first pressure is 40 to 60Pa.

Sublimation steps. In the freezing condition, the pressure is lowered from the first pressure to the second pressure for sublimation, in the sublimation process, the temperature gradually rises. Most of the moisture is removed in the process. The secondary pressure here is 20 ~ 30Pa. During the sublimation process, which takes place under a high vacuum, the contents of the container must be kept frozen during the pressure reduction process to prevent overflow of the container. When the pressure in the box falls to 30Pa and below -65℃, the ice begins to sublimate, and the sublimated water vapor forms ice crystals in the condenser. In order to ensure the

sublimation of ice, it is preferred to open the heating system, heating the shelf, the temperature reaches the eutectic point, -28℃ ~ -10℃. Generally speaking, the lower the air pressure is, the more favorable it is to sublimation and water vapor escape, but the invention finds that the air pressure below 15Pa is unfavorable to sublimation. Therefore, the sublimation pressure of the invention is generally 15Pa to 80Pa. During the sublimation process, the sublimation step is terminated when the temperature reaches -28℃ ~ -10℃.

Re-drying steps. Raise the temperature to 30 ~ 35℃, and dry again in this process until the moisture content is below 9wt %, preferably below 5-9wt %. At this time, the water vapor pressure on the solid surface decreases in different degrees, and the drying rate decreases obviously. In this stage should be properly raised shelf temperature, in order to facilitate the evaporation of water, is generally heated shelf to 30-35℃, until the sample temperature and shelf temperature coincide to dry so far. The moisture content of the freeze-dried samples should be controlled below 5-9wt %, preferably below 7wt %. In some implementations, moisture is controlled between 5 and 7wt %.

3.2.3. The main methods of technology for obtaining an extract of American ginseng

Brief process flow

1. Original medicinal materials were selected, and non-medicinal parts were removed;

2. Rinsing and crushing: rinsing the good American ginseng and crushing it to 8 ~ 32mm;

3. Drying: Freeze drying technology was used to dry American ginseng until the water content of the original medicine was below 5-9wt%.

4, coarse grinding: the American ginseng is first used universal grinding to 60 ~ 100 mesh, and then ball grinding to 100 ~ 500 mesh, to get American ginseng powder;

5, broken wall crushing: The coarse powder of Panax quinquefolium was put into the airflow pulverizing machine. Under the feeding pressure of 0.5 ~ 0.9 MPa, the frequency of 35 ~ 45Hz, the air pressure difference of 0.2 ~ 0.8 MPa, the water content of the material of 5 ~ 9wt% and the mesh size of 100 ~ 500 mesh conditions, the pulverized to 300 ~ 1500 mesh, the wall breaking powder was obtained.

6. Ultrafine powder encapsulation: ultrafine powder is pelleted without adding any auxiliary materials or additives. The forming pressure is generally about 70 ~ 130, the maximum pressure is 200bar, the spiral speed is 80rpm, the closing speed is 20rpm, and the whole grain speed is 100rpm, and finally 30 ~ 200 mesh wall breaking particles of TCM decoction pieces are obtained.

7. Total mixing: Put American ginseng particles into a 3d motion mixer motion mixing machine and mix well to ensure that the Angle of repose is less than 40°.

8. Filling: 1 ~ 3g small bag packaging, easy to take and carry.

Fig. 4 – Pelletizer.

Fig. 5 - 3d motion mixer.

Freeze drying technology. The above step 3 drying is to freeze the material below freezing point, turn the water into ice, and then remove the ice by turning it into steam under a higher vacuum. The specific steps are as follows:

(1) For freezing, the materials to be freeze-dried shall be cooled to about 2.5 ℃ with appropriate cooling equipment, and then placed in a freeze-dried box cooled to about -38℃(40Pa). Close the drying oven, quickly into the refrigerant (freon, ammonia), so that the material frozen, and keep for a long time, to overcome the solution undercooling phenomenon, so that the product completely frozen, can be sublimated.

(2) Sublimation is carried out under a high vacuum. In the process of pressure reduction, the items in the box must be kept frozen to prevent overflow of the container. After the pressure in the box drops to a certain extent, open the vacuum pump (or vacuum diffusion pump), and when the pressure drops to 25Pa and below -65℃, the ice begins to sublimate, and the sublimated water vapor forms ice crystals in the condenser. In order to ensure the ice sublimation, the heating system should be turned on to heat the shelf and continuously supply the heat needed for ice sublimation. The temperature reaches the eutectic point, -28℃ ~ -10℃ (varies according to different products).

(3) then dry in the sublimation stage, a large number of ice sublimation, at this time to be dried product temperature should not exceed the lowest eutectic point, in order to prevent the production of rigid block or product appearance defect, in this stage shelf temperature is usually controlled between ±10℃. The moisture removed in the redrying stage of the product is combined moisture, and the vapor pressure on

the solid surface decreases in different degrees, and the drying speed decreases obviously. Below the premise that assures product quality, shelf temperature should be raised appropriately inside this stage, in order to facilitate the evaporation of moisture, it is to put shelf heating to 30 ~ 35℃ commonly, until product temperature and shelf temperature coincide to achieve dry so far. The moisture content of lyophilized samples was controlled below 5 ~ 9wt%.

Selection of ultrafine grinding method of American ginseng. First, universal grinding was used to 60 ~ 100 mesh, and then ball grinding was used to grind the raw materials of Chinese medicinal materials into 100 ~ 500 mesh powder, with a yield of more than 97%. Airflow grinding technology was used for three times of grinding, with feed pressure of 0.6mpa, frequency of 37Hz and air pressure difference of 0.6mpa. Water cut 5 ~ 9 wt.%, feed raw materials number under the condition of 100 ~ 500 purpose of airflow pulverization and crushing to 300 ~ 1500 mesh, the wall powder, yield of around 96% (not directly after a meal to break the wall crushing yield only at around 84%), improve the difficult to crush material wall broken rate, improve product yield, to save the original Chinese herbal medicine raw materials.

3.3. Research on quality control and evaluation methods of broken Chinese medicine decoction pieces

Qualitative identification methods. DNA gene identification technology. Due to the wall yinpian has lost its TCM yinpian appearance identification properties, and the pure from the Angle of the chemical composition is difficult to

identify and evaluate the most TCM of authenticity and confused, so the relatives of the plant species and confusion of objects in identification of DNA gene identification technology was introduced in the evaluation of TCM yinpian homogeneity of the broken wall.

DNA barcoding is a technique for species identification of TCM using a standard and relatively short DNA fragment in the genome. The gene spacer sequences between 18S-5.8S-28s rDNA, called ITS, are highly conserved in evolution, and their conserved nature is basically consistent within species, with obvious differences between species. Sequence analysis after amplification can be used for the identification of broken decoction pieces of TCM and ITS related species. The similarity of ITS sequence between traditional decoction pieces of Honeysuckle and Fructus evodia officinalis and ultramicro decoction pieces is greater than 99%, so ITS sequence analysis can be effectively used for identification of ultramicro decoction pieces, which is the development of identification methods of Traditional Chinese medicine 178.. Shun-xiang li, etc. 180. will be 14 batches of kudzu root (kudzu 4 batch, kudzu 10 group) after samples made of superfine slices to analyze ITS sequence and build the system tree, found sequence, kudzu powder, kudzu powder ge ge sequence sequence identity are 81%, 98% and 62% respectively, with kudzu powder ge significant difference between ITS sequence, a maximum length difference of up to 180 Bp could be used to distinguish pueraria pueraria from pueraria superfine decoction pieces, indicating that ITS sequence analysis could be used for variety identification and quality control of pueraria superfine decoction pieces. At present, the manufacturers of broken Chinese

medicine decoction pieces have carried out homogeneity research on evaluation of broken Chinese medicine decoction pieces by gene identification technology. It is believed that with the continuous improvement of this technology, the application of DNA gene identification technology in qualitative identification of broken Chinese medicine decoction pieces will be more and more extensive.

TLC is one of the most commonly used methods in qualitative identification of TCM. Generally, reference substance with known active components or the same extract of reference material are selected for comparison. After TLC expansion and color development, the identification is carried out by observing whether the sample and reference substance have the same spots at the corresponding position. Li Shouxin et al. [181] prepared test solution and control solution from the reflux extract of red ginseng ultrafine powder and the mixture of ginsenosides Rg1, Re and Rb1, respectively, and conducted TLC identification. The results showed that the test sample and the mixed control sample had the same spots on the corresponding positions. TLC was used to identify ultrafine decoction pieces of *Fritillaria thunbergii*, tempeh, fructus trichosanthis, astragalus complandii seeds, litchi seeds, lotus seeds, long pepper and other extracts from the original medicinal materials. It was found that the ultrafine decoction pieces of TCM had the same chemical composition with the original medicinal materials, and TLC method could be used to identify these ultrafine decoction pieces [182].

TCM is mostly used as compound medicine, so there are some attempts to make compound ultra-fine yinpian after processing each flavor, and compare it with traditional compound and reference substance by TLC. Tonifying Yang also five

decoction compound superfine slices of water immersion and traditional yinpian decocting medicinal broth and compound of angelica sinensis, radix paeoniae rubra, rhizoma ligustici wallichii, safflower, peach kernel five taste of traditional Chinese medicine herbs and reference substance of TLC identification, found that the compound superfine yinpian, traditional one, the control medicinal materials, reference substance in the corresponding position have the same spots, and ultramicro yinpian spots of the main effective components in water immersion Traditional soups are more obvious and clear [34].

HPLC fingerprint can reflect the overall chemical composition characteristics of TCM and has been widely used. This technology is mainly used for homogenization and stability analysis of different batches of raw materials, component stability analysis before and after wall breaking process, and intra-batch and inter-batch homogenization and stability analysis of finished products of wall broken decoction pieces.

Ciic pharmaceutical group respectively for gold hair pin caulis dendrobii [183], red ginseng, astragalus membranaceus [184], of notoginseng [185] and salvia miltiorrhiza [186], radix scrophulariae, dangshen [187] and [186] of cassia seed [188] wall-breaking yinpian, broken wall powder (intermediate) and traditional yinpian (raw material) in the HPLC fingerprint study, results wall yinpian, breaking wall powder (intermediate) There were high similarity of fingerprints and good correlation of main components between different batches of broken decoction pieces and traditional decoction pieces (raw materials), indicating that the chemical components of the medicinal materials were basically stable and consistent before

and after breaking the wall, and the stability and uniformity of samples between batches were also good.

Ju Aihua et al. [189] established the fingerprint of Gardenia jasminoides from 10 batches of different producing areas by comparing the differences between the fingerprints of ultrafine powder and ordinary powder with jasminoside and isojasminoside as control materials by HPLC method. CAI Ping et al. established HPLC fingerprint of ultramicro decoction pieces of red ginseng. With ginsenoside Rg1 as reference, 11 common peaks were established, which can provide basis for the identification and quality control of ultramicro decoction pieces of red ginseng.

The composition of TCM is complex, and the establishment of multiple fingerprints can reflect its material basis and identification characteristics more comprehensively by using multiple analysis methods. Xiao-yan feng etc. 13 batches of different origin of superfine powder of radix isatidis herbs fingerprint of the water soluble constituents and the amino acid respectively before column derivatization fingerprint study, and established the second yuan fingerprint common model, the calibration of adenosine, table to according to the characteristic peak of spring and 15 kinds of amino acids, good separating degree, in the chromatogram peak characteristics significantly, for the comprehensive control board blue The quality of root ultrafine powder was provided.

However, the current fingerprint of TCM is not mature, so it is impossible to accurately evaluate the relationship between components and pharmacodynamics of TCM wall-breaking decoction pieces. On this basis, it will become the future

development direction to explore the spectroscopic-effect relationship of TCM wall-breaking decoction pieces and reveal its pharmacological mechanism.

Content determination of single or several pharmacodynamic evaluation index components. HPLC method has the characteristics of high sensitivity, fast analysis speed and high separation efficiency, and is mainly used for the determination of single component in broken Chinese medicine decoction pieces. Wang Yun et al. [190] used HPLC-ELSD method to determine the content of astragaloside IV in astragalus membranaceus broken decoction pieces after heating and reflux extraction. This method is simple and quick, and can be used as a method to determine the content of astragaloside IV in Astragalus membranaceus broken decoction pieces. Zhan Ruoting et al. [191] determined the contents of ginsenosides Rg1, Re and Rb1 in the wall-breaking powder of red ginseng by HPLC and investigated the transfer rate of ginsenosides, which proved that the amount of ginsenosides dissolved in the wall-breaking powder of red ginseng was higher than that of red ginseng. Liu Min et al. [192] established HPLC method for the content determination of saponins in panax notoginseng broken wall powder. The method was simple and feasible, with stable results and good repeatability, which could provide a basis for the formulation of quality standard of panax notoginseng broken wall powder.

The principle of U(H) PLC is basically the same as THAT of HPLC, but the analysis speed is faster, the separation degree and sensitivity are higher, and it has obvious advantages in the analysis of broken Chinese medicine decoction pieces with complex components. Cheng Hui et al. [193] used UPLC method to

simultaneously determine the contents of rosinic acid and isoazine picidine in zhuangjie wind ultrafine powder, which can provide reference for the quality control of Zhuangjie wind ultrafine powder.

GC method can separate multi-component mixtures, and provide quantitative data, fast and convenient, suitable for the analysis of broken Chinese medicine decoction pieces containing volatile oil and other volatile components. After dendrobium nobile was crushed into fine powder and superfine powder, the content of dendrobiine in fine powder and superfine powder was determined by GC method. The results showed that the content of dendrobiine in fine powder and superfine powder was 0.42% and 0.35% respectively, the content of dendrobiine in ultrafine powder was lower than that in fine powder, which was thought to be caused by the increase of temperature or specific surface area of powder by grinding. Attention should be paid to the method, temperature of ultrafine grinding and the packaging and storage conditions of ultrafine decoction pieces 194.. The content of oleic acid and linoleic acid in ganoderma lucidum spore powder were determined by GC in the range of 0.4014 ~ 2.0072 μg and 0.1330 ~ 0.6648, respectively the method is simple, rapid, reliable and reproducible, and can be used for the quality control of broken ganoderma spores powder [195].

Determination of the content of active ingredient groups. UV method is a common method for the determination of total polysaccharides, total triterpenoids, total flavonoids and other total active ingredients. Jin Ziming et al. [196] measured the content of polysaccharides in superfine powder and ordinary powder by UV method at 490 nm and compared their solubility. The results showed that the average

content of polysaccharides in superfine powder of Codonax codonax in 3 batches was 23.47%, which was 1. 25 times that of ordinary powder, and had a higher solubility. Chen Guan-zhou et al. [197] determined the content of total triterpenoids in the extract of a broken ganoderma lucidum spore powder by UV method with ursolic acid as control substance,5% vanilla-acetic acid and perchloric acid as chromogenic agent, and found that the content of ursolic acid was in the range of 14 ~ 15 In the range of μg, the linear relationship with absorbance is good, and the method is simple to operate, fast to test, high stability, good repeatability. Ju Aihua et al. [53] took 500 nm as the detection wavelength, measured the dissolution degree of total flavonoids in The Mongolian medicine Citropteris communis by UV method and compared it, and confirmed that the dissolution amount of total flavonoids in the superfine powder of Citropteris communis communis is about 1.2 times that of the ordinary powder, and the dissolution rate is significantly improved.

Content determination of safety evaluation index components. According to the current data analysis, the safety and quality control methods of broken Chinese medicine decoction pieces mainly include determination of heavy metals and harmful elements by ICP-MS; Determination of pesticide residues by GC; Determination of sulfur dioxide residue by titration or HPLC; Determination of aflatoxin by HPLC.

Inductively coupled plasma mass spectrometry (ICP-MS) is a highly sensitive technique for the determination of inorganic elements and isotopes, which can be used for the determination of heavy metals in broken Chinese medicine decoction pieces. Determination of Pb, As, Hg, Mn, Cr of 33 batches of broken Ganoderma

spores by ICP-MS The contents of Cd and Ni in some ganoderma spores before and after wall breaking were compared. It was found that the contents of Pb, As, Mn, Cr and Ni increased significantly after wall breaking, suggesting that metal devices contacted during wall breaking were one of the causes of heavy metal pollution [198]. Xu Jing determined by icp-ms method [199] such as raw material, the vibration upon maturity of lingzhi only worn out wall, airflow pulverization upon maturity of lingzhi only broken in upon maturity of lingzhi only V, Cr, Mn, Sn, Hg and other 16 kinds of inorganic element content, after found broken ganoderma lucidum spore elements such as Cr, Fe, Ni, Co in the content increased significantly, but the wall samples than air vibration grinding broken wall led sample More heavy metals were added.

The content control of heavy metals, agricultural residues and aflatoxin is the key and difficult point of quality control of broken Chinese medicine decoction pieces. In recent years, Chinese medicine wall broken decoction pieces production enterprises pay more attention to the content determination of heavy metals, agricultural residues and aflatoxin in Chinese medicine wall broken decoction pieces, and some achievements have been made. It is necessary to strengthen the research in this aspect in the future.

4. Conclusions to section 3

TCM decoction pieces have been used for thousands of years in China and have made great contributions to the reproduction and survival of the Chinese nation. However, due to the disadvantages of inconvenient decocting and unstable quality,

the application of traditional Chinese medicine is far less extensive than that of western medicine in recent years. The development of broken Chinese medicine decoction slices is helpful to realize the modernization of Chinese medicine and carry forward Chinese medicine culture.

As the future development direction of TCM decoction pieces, although it started late, it highlights the broad market value with its unique advantages. It can be seen that with the further in-depth research on TCM wall-breaking decoction pieces, it will provide a scientific research basis for the development and application of TCM wall-breaking decoction pieces, and TCM wall-breaking decoction pieces will definitely become the development direction of TCM decoction pieces in the future and the main trend of promoting the internationalization of TCM decoction pieces. American ginseng is a rare Chinese medicine, and its nutritional value can be maximized by breaking the wall of TCM, which provides a new research idea for the industrial development of American ginseng.

Conclusion

At present, TCM has occupied an important position in the field of anti - inflammation. In the process of prevention and treatment of COVID-19, TCM preparations such as Lianhua Qingwen preparation and Qingfei Didutang have become important therapeutic methods to suppress the deterioration of patients' condition and enhance the immune function of susceptible patients due to their good antiphlogisticand preventive effects and play a leading role in the recovery of COVID-19 patients and rehabilitation after discharge. It can be seen that Chinese medicine has great development potential in the field of antiphlogistic.

New model in this study, the use of biological zebrafish as the carrier, zebrafish as a complete life body, can objectively and comprehensively, systematically to evaluate the antiphlogisticactivity of traditional Chinese medicine and its effective components, and realize the fast and effective high-throughput screening, as well as antiphlogisticmechanism of TCM research provides a good model. Three inflammatory models: LPS, tail cutting and IBD were used to study the antiphlogisticeffects of *Panax quinquefolium*. Firstly, the safety of *American ginseng* was evaluated, and the safe concentration was selected. Subsequently, the antiphlogisticeffect of *American ginseng* on the inflammatory zebrafish model was observed in all model groups. This experiment for the first time the experimental inflammatory bowel disease model to verify its pharmacological activity, through the antiphlogisticaction of two indicators to examine *American ginseng*, let drugs in a pathological state down to study the antiphlogistic effect, make its

antiphlogisticeffects more convincing, for subsequent research on the antiphlogistic drugs provides a new train of thought, and laid a solid foundation to the research of *American ginseng* antiphlogistic mechanism.

In the third part of the study, the possibility of *Panax quinquefolium* as a TCM was explored. With the increase of market demand and the extreme shortage of TCM resources, TCM dosage forms (pills, powder, ointment, Dan, decoction, etc.) cannot meet the needs of modern clinical and patients. The development of broken Chinese medicine decoction slices is helpful to realize the modernization of Chinese medicine and carry forward Chinese medicine culture. American ginseng is a rare Chinese medicine, and its nutritional value can be maximized by breaking the wall of TCM, which provides a new research idea for the industrial development of *American ginseng*. The full use of zebrafish model will provide new ideas and methods for the study of antiphlogistic TCM and promote the development of antiphlogistic TCM. Meanwhile, zebrafish model will have a broader application prospect in the field of antiphlogistic TCM.

Due to the impact of COVID-19, the research period was greatly shortened, so the antiphlogistic mechanism of *American ginseng* has not been further verified. Therefore, molecular biological exploration can be conducted around the IBD pathway to explore its antiphlogisticmechanism through qPCR technology. The research on the technique of breaking the wall of American ginseng has not been put into production line. The follow-up work of this subject will continue to be improved.

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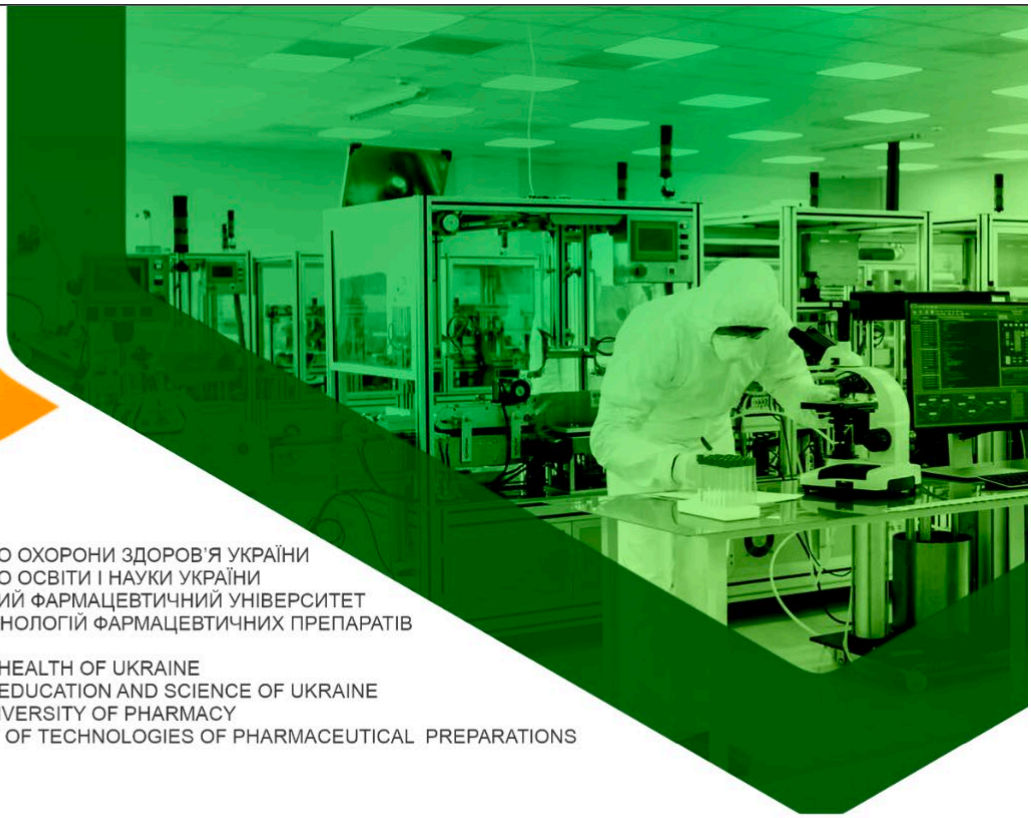

МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ
МІНІСТЕРСТВО ОСВІТИ І НАУКИ УКРАЇНИ
НАЦІОНАЛЬНИЙ ФАРМАЦЕВТИЧНИЙ УНІВЕРСИТЕТ
КАФЕДРА ТЕХНОЛОГІЙ ФАРМАЦЕВТИЧНИХ ПРЕПАРАТІВ

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ANTI-INFLAMMATORY EFFECT OF AMERICAN GINSENG ON ZEBRAFISH TAIL AMPUTATION INFLAMMATION MODEL

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Panax quinquefolium Linn. (American Ginseng or Ginseng) is a perennial herb of Panax genus of Araliaceae, native to the eastern part of North America, mainly from Canada and the United States, which has been introduced and cultivated in China for many years [1-3]. American ginseng contains a variety of effective ingredients, with anti-inflammation, anti-cancer, blood pressure, blood lipids, anti-fatigue and other pharmacological activities.

Zebrafish are already widely used in drug screening and share about 87 percent of their homologous genes with humans. Compared with other model animals, zebrafish species are stable and individual differences are small. Small size, strong resistance to pathogens, easy to large-scale breeding, low maintenance costs; It has strong reproductive capacity and large spawning capacity, and can realize forward genetic research based on phenotype. The short reproductive period can obviously shorten the research period.

Take an appropriate amount of dried American ginseng root produced by Wendeng, cut it with scissors, weigh it, add 8 ml of 50% ethanol to each 1g of dried American ginseng, ultrasonic extract for 1 h at 50 ℃ and 40 kHz, centrifuge the extract at 20 ℃ and 11000 rpm for 20 min, take the supernatant, add it into an ampoule bottle, and dry it at 60 ℃ under a dryer to obtain American ginseng extract (AGE).

The experiment verified the anti-inflammatory effect of American ginseng by causing an inflammatory state to zebrafish and using American ginseng extract to alleviate the inflammatory effect. We used the transgenic zebrafish line TG (zlyz: EGFP) expressing enhanced green fluorescent protein (EGFP) in immune cells to evaluate the anti-inflammatory effect of American ginseng from the perspective of phenotype. Zebrafish tail amputation inflammation model is a traumatic inflammation model. The treatment of zebrafish tail amputation can induce local damage to zebrafish tail and promote the immune response of zebrafish immune cells [4]. The anti-inflammatory effect of American ginseng was evaluated by measuring the local fluorescence intensity through the aggregation of immune cells at the broken tail.

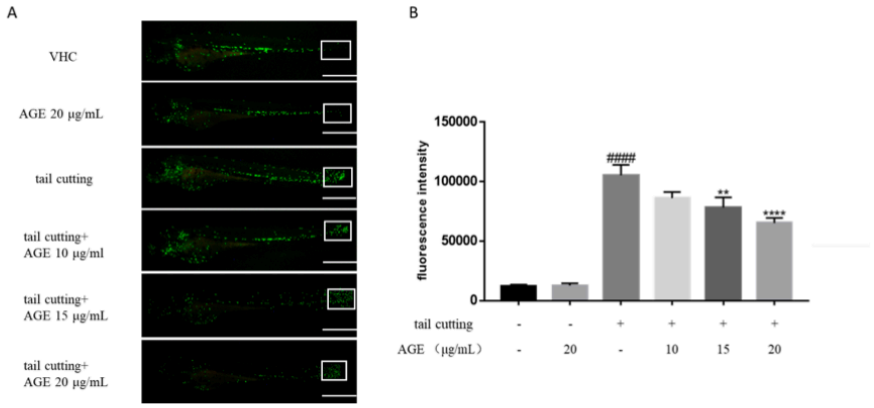

FIG. 1 Change in the intensity of local fluorescence depending on the concentration of the ethanol extract of American ginseng root.

(A) The aggregation of inflammatory cells at tail termination was traced by transgenic zebrafish with green fluorescence labeling. (B) Quantitative analysis of the fluorescence intensity of inflammatory cells in the same region. #### $p < 0.0001$ compared with control group, the ****$p < 0.01$, $p < 0.0001$ compared with the tail group.

Compared with blank control group, inflammatory cells labeled with green fluorescence were significantly aggregated in the tail-cut group. The number of inflammatory cells in the tail region of juvenile zebrafish was significantly decreased in AGE group compared with model group at 10, 15 and 20μg /mL AGE after 12 h treatment. The number of inflammatory cells decreased with the increase of AGE concentration. Subsequent experiments will further study the anti-inflammatory effects of Panax quinquefolium by qPCR and other methods from the perspective of mechanism.

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Involvement of dopamine signaling pathway in neurodevelopmental toxicity induced by isoniazid in zebrafish

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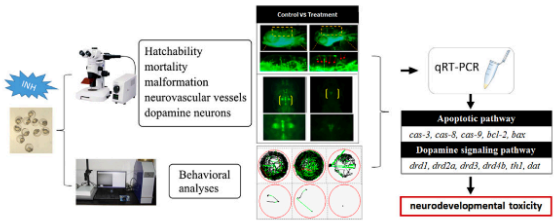
HIGHLIGHTS

- INH impaired the development of vasculature and reduced the behavior of zebrafish.
- INH induced brain apoptosis and increased the expression of apoptosis related genes.
- INH inhibited dopamine neuron development and the expression of dopamine related genes.

GRAPHICAL ABSTRACT



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ABSTRACT

Aims: This study evaluated the neurodevelopmental toxicity of isoniazid (INH) in zebrafish embryos and the underlying mechanism.
Methods: Zebrafish embryos were exposed to different concentrations (2 mM, 4 mM, 8 mM, 16 mM, 32 mM) INH for 120 hpf. During the exposure period, the percentage of embryo/larva mortality, hatching, and morphological malformation were checked every 24 h until 120 hpf. The development of blood vessels in the brain was observed at 72 hpf and 120 hpf, and behavioral capacity and acridine orange (AO) staining were measured at 120 hpf. Alterations in the mRNA expression of apoptosis and dopamine signaling pathway related genes were assessed by real-time quantitative PCR (qPCR).
Results: INH considerably inhibited zebrafish embryo hatching and caused zebrafish larval malformation (such as brain malformation, delayed yolk sac absorption, spinal curvature, pericardial edema, and swim bladder defects). High concentration of INH (16 mM, 32 mM) even induced death of zebrafish. In addition, INH exposure markedly restrained the ability of the zebrafish autonomous movement, shortened the length of dopamine neurons and inhibited vascular development in the brain. No obvious apoptotic cells were observed in the control group, whereas considerable numbers of apoptotic cells appeared in the head of INH-treated larvae at 120 hpf. PCR results indicated that INH significantly raised the transcription levels of *caspase-3*, *-8*, *-9*, and *bax* and significantly decreased *bcl-2* and *bcl-2/bax* in the

Abbreviations: : isoniazid, (INH); hour post-fertilization, (hpf); acridine orange, (AO); green fluorescent protein, (GFP); B-cell lymphoma 2, (*bcl-2*); BCL2 Associated X, (*bax*); tyrosine hydroxylase, (*th1*); dopamine transporter, (*dat*); vesicular monoamine transporter, (*Vmat*); aromatic L-amino acid decarboxylase, (AADC); prefrontal cortex, (PFC).

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zebrafish apoptotic signaling pathway. INH also markedly decreased the genes related to dopamine signaling pathway (*th1*, *dat*, *drd1*, *drd2a*, *drd3*, and *drd4b*).
Conclusions: Experimental results indicated that INH had obvious neurodevelopmental toxicity in zebrafish. Persistent exposure to INH for 120 h caused apoptosis, decreased dopaminergic gene expression, altered vasculature, and reduced behaviors.

1. Introduction

In the clinical treatment of tuberculosis, isoniazid (INH) is a first-line anti-tuberculosis drug with strong germicidal effect against *Mycobacterium tuberculosis* (WHO, 2019). With the extensive use of INH, its adverse reactions in clinical practice have also gradually appeared. In addition to hepatotoxicity, it can also cause peripheral neuritis and central nervous system complications (Erwin et al., 2019; Ruan et al., 2018), which manifest as coma, tremor in the hands and feet, nerve disorders, numbness, and convulsions (Skinner et al., 2015). The level of toxicosis is dose-dependent (Zuur et al., 2018). Congenital tuberculosis is usually caused by transmission from maternal circulation to fetal circulation or from aspiration and ingestion of amniotic fluid or maternal blood. In pregnant women with tuberculosis, the risk of fetal infection is very high (Benjamin et al., 2019).

It has been reported that drug treatment in pregnant women with tuberculosis (rifampin and INH combined for 3 months) can greatly reduce the probability of tuberculosis in newborns (Hamada et al., 2018; Pease et al., 2018). The treatment of tuberculosis during pregnancy is disputed (Mathivha et al., 2017). For example, INH can penetrate the placental barrier, making the blood drug concentration in amniotic fluid higher than that in maternal blood, leading to intrauterine growth retardation, low birth weight (Tamura et al., 2019), and increased risk of death (Mittal et al., 2014). Bharathi et al. (2012) gave anti-tuberculosis drugs to pregnant rats and continued the observation of newborn pups. They found that the therapeutic dose of INH also inhibited the cognitive ability of newborns. However, there are few reports on the toxic mechanism of INH affecting embryonic neurodevelopment.

As an ideal vertebrate evaluation model, zebrafish have been extensively used in studies of developmental toxicity. Its genes are 87% homology with human genes (Tsang et al., 2017). In addition, the volume of zebrafish is small, the embryo is transparent, and the developmental process of the central nervous system can be dynamically observed using fluorescent proteins to specifically label zebrafish nerve cells. Dopamine neurons in zebrafish start to form at 24 h of development and start to differentiate at 30 h of development. Zebrafish are an ideal model animal to investigate neurodevelopment, behavior integration mechanisms, and neurotoxicology (Du et al., 2016; Murilo et al., 2019; d'Amora et al., 2018).

Apoptosis and dopamine pathway play important roles in neurotoxicity. Cao et al. found that Involvement of caspase activation in hoiamide A-induced neurotoxicity (Cao et al., 2015). The current literature increasingly supports the notion that polymyxin-induced neurotoxicity is largely related to cell death via apoptosis (Dai et al., 2017, 2019). Ares Santos, S et al. studied that methamphetamine induced gene inactivation of dopamine receptor, and reduce the level of dopamine in mice by reducing the activity of dopamine transporter, thus leading to neurotoxicity (Ares Santos, S., 2013). Decreased tyrosine hydroxylase (*th*) activity leads to abnormal dopamine pathway and neurotoxicity (Granado, N., 2010). Based on the advantages of zebrafish and previous studies, we used zebrafish embryos as a model to investigate the neurodevelopmental toxicity and the underlying mechanism of INH through apoptosis, dopamine pathway, behavior test and other indicators. The aim of this study is to provide a basis for further toxicity research and clinical application of INH.

2. Materials and methods

2.1. Reagents and instruments

INH was purchased from Sigma (CAS No. 54-85-3; >99% purity, St. Louis, MO, USA). The required concentrations of INH were prepared using zebrafish culture water. Zebrafish embryo culture water contained 5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$ and 0.16 mM $MgSO_4$. Tricaine was purchased from sigma (CAS No. 886-86-2; >98% purity). Tricaine was prepared with purified water to a 0.16% solution and placed in a 4 °C freezer for future use. Other chemical reagents in this study were all analytic grade.

2.2. Zebrafish

This study used the wild-type AB line and the following transgenic zebrafish: the Tg (vmat2:GFP) line with green fluorescent protein (GFP)-labeled dopamine neurons and the Tg (fli1:GFP) line with green fluorescent protein-labeled vasculature. Zebrafish were maintained under a standard temperature of 28 °C, 14 h of light and 10 h of darkness per day, and were fed saltwater shrimp twice a day. When eggs were used, healthy and sexually mature zebrafish were placed in a feeding box at a female to male ratio of 1:1 or 1:2. Mating and spawning were ended at 8 o'clock in the morning on the next day, and embryos were collected at 10–11 o'clock. Embryos were washed with culture water 3 times, disinfected using 0.1% methylene blue (Collymore et al., 2014), transferred into zebrafish embryo culture water, and cultured in a 28 °C constant temperature- and light-controlled incubator (light for 14 h/dark for 10 h). All experiments were conducted in accordance with the ethical standards guidelines of Biology Institute, Qilu University of Technology.

2.3. INH exposure

At 4 h post fertilization (hpf), the embryos were examined under a dissecting microscope (Olympus, Tokyo, Japan) and those had developed normally to the blastula stage were selected for subsequent experiments. In the experiment, the control group used embryo culture water, and the various concentrations of INH prepared using embryo culture water. Randomly place 30 embryos per well into 6-well plates, and each well is given 5 mL of INH solution of different concentrations (2, 4, 8, 16 and 32 mM). After administration, light-controlled culture was carried out in incubator, the solution was changed every 24 h, and the dead embryos were discarded everday, continuous exposure for 120 h. Experiments were repeated three times.

2.4. Effects of INH on mortality, hatching, and morphology of zebrafish

The number of dead zebrafish embryos at different concentrations was recorded every 24 h using a stereo fluorescence microscope (SZX16, Olympus, Tokyo, Japan). The numbers of hatched and deformed embryos were recorded to calculate the percentage of larvae cumulative mortality (24 hpf-120 hpf), the percentage of larvae hatched (48 hpf-120 hpf), and the percentage of larvae malformation (48 hpf-120 hpf). Determination of death was based on egg coagulation and cardioplegia.

The development conditions of 10 randomly selected fish in each group were observed at 24–120 hpf. Larvae were anesthetized using tricaine, and morphological changes was photographed and recorded under a Stereo microscope (AXIO Zoom. V16, ZEISS, Oberkochen, Germany) to observe and record all development indicators, including morphological changes in the brain, presence of pericardial edema, delayed yolk sac absorption, spinal curvature, absence of swim bladders and shortening of the body length.

2.5. Behavioral analyses of zebrafish

Behavioral analyses of zebrafish after 120 h of INH exposure were performed. The zebrafish larvae from each group were collected, cleaned in embryo culture water and placed in 48-well plates, exposed to the embryo culture water with 1 larva in each well (8 replicates). Larvae were placed in a dark box in a zebrafish behavioral analysis apparatus (Viewpoint Life Sciences, Lyon, France). The recording area was set up by the ZebraLab software (Viewpoint Life Sciences, Lyon, France) to ensure that the swimming area of each zebrafish (i.e. the size of the aperture) was within the recording area. After zebrafish were allowed to adapt for approximately 15 min, behavioral tracking was performed. The movement track of each group of larvae in 40 min was collected by software. Recording was performed for every 60 s. After obtaining the data, the data were statistically analyzed, including total swimming distances, total average movement speed, average movement speed at each time period, and movement distance at the high, medium, and low movement conditions.

2.6. Acridine orange (AO) staining

AO is a nucleic acid-selective metachromatic dye. It emits green fluorescence after inserting into DNA. AO can permeate apoptotic cells and bind DNA, whereas AO cannot permeate healthy cells. Therefore, it is extensively applied in the detection of apoptotic cells in zebrafish (Monaco et al., 2017). At 120 hpf, larvae were washed with PBS 2 times, incubated with 5 μg/mL AO at 28 °C in the dark for 20 min, and washed thoroughly with PBS 3 times (Jin et al., 2019). Eight individuals from each group were randomly selected for visual observation and image acquisition. The experiments were repeated three times. Images of the brain on the dorsal side of anesthetized zebrafish were collected under a fluorescence microscope (AXIO Zoom. V16, ZEISS, Oberkochen, Germany) and processed using Image-Pro Plus 5.1 software (Media Cybernetios Inc, Rockville, MD, USA).

2.7. Evaluation of the development of vasculature in the brain of zebrafish

The transgenic zebrafish Tg (fli1:GFP) were used to evaluate the development of neural vasculature. Tg (fli1:GFP) zebrafish larvae at 72 hpf and 120 hpf were anesthetized, and 8 larvae in each group were randomly selected. Zebrafish heads were photographed under a light field and a fluorescence field to observe the development of vasculature in the brain of zebrafish.

2.8. Evaluation of the development of dopamine neurons in zebrafish

Tg (vmat2:GFP) zebrafish larvae at 72 hpf and 120 hpf were anesthetized, and 8 larvae in each group were randomly selected. Zebrafish heads (dorsal side) were photographed under a fluorescence microscope (AXIO Zoom. V16, ZEISS, Oberkochen, Germany) to observe the development of dopaminergic neurons in brain region. The length of dopamine neurons in the raphe nuclei was analyzed by using Image-Pro Plus 5.1 software (Media Cybernetios Inc, Rocjvile, MD, USA).

2.9. Detection of gene expression levels using real-time fluorescence quantitative PCR

30 zebrafish larvae at 120 hpf were collected in each group. Total tissue RNA was extracted by using the tissue total RNA isolation kit (Vazyme, Nanjing, China). The RNA samples of each group were reverse transcribed into cDNA. The expression levels of apoptosis-associated genes and dopamine signaling pathway-associated genes in zebrafish were detected using AceQ® qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) in a LightCycler 96 real-time fluorescence PCR machine (Biorad, CA, USA).

The settings for the real-time fluorescent PCR amplification reaction were as follows: The first cycle is preincubated at 95 °C for 300 s, followed by a 3-step amplification: denaturation at 95 °C for 10 s, and annealing at 60 °C for 45 cycles. Perform a cycle at 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s to generate a melting curve, and finally cool at 37 °C for 15 s. The fluorescence signals after cyclic annealing were collected. Real-time PCR was used to detect gene expression levels of apoptosis and dopamine signaling pathways, and β-actin was used as a housekeeping internal control gene primer. The primers are designed and synthesized by Generay Biotech Co., Ltd. (Shanghai, China) and their sequences are showed in Table 1.

2.10. Statistical analyses

Data of each group were expressed as means ± standard error of mean (SEM). The significant differences were determined by using one-way analysis of variance (ANOVA) tests. The number of independent experiments is represented by n. *$p < 0.05$ was considered significant, and **$p < 0.01$ was considered highly significant.

3. Results

3.1. Effects of INH on the zebrafish mortality

There was no significant difference in mortality of zebrafish between the INH group (2 mm, 4 mm and 8 mm) and the control group at 120 hpf. The mortality of zebrafish larvae in the 16 mM group was as high as 46.15% and was significantly differential at 96 hpf. Many zebrafish died in the 32 mM group at 120 hpf, and the mortality was close to 90%. INH induced zebrafish death in a concentration dependent manner (Fig. 1A).

3.2. Effects of INH on the hatchability and developmental morphology of zebrafish embryos

The hatchability and morphology development are important indicators for the evaluation of zebrafish development (Al-Kandari H et al., 2019). At 48 hpf, zebrafish larvae were hatched in the control group and low concentration group (2 mM), but not in the

Table 1
The gene Primers for Quantitative Real-time PCR.

Gene	Primer orientation	Nucleotide sequence
β-actin	Forward	5′-AGAGCTATGAGCTGCCTGACG-3′
	Reverse	5′-CCGCAAGATTCCATACCCA-3′
caspase-3	Forward	5′-CCGCTGCCCATCACTA-3′
	Reverse	5′-ATCCTTTCACGACCATCT-3′
caspase-8	Forward	5′-CCAGACAATCTGGATGAACTTTAC-3′
	Reverse	5′-TGCAAACTGCTTTATCTCATCT-3′
caspase-9	Forward	5′-CTGAGGCAAGCCATAATCG-3′
	Reverse	5′-AGAGGACATGGGAATAGCGT-3′
bax	Forward	5′-GGCTATTTCAACCAGGGTTCC-3′
	Reverse	5′-TGCGAATCACCAATGCTGT-3′
bcl-2	Forward	5′- TCACTCGTTCAGACCCTCAT -3′
	Reverse	5′- ACGCTTTCCACGCACAT -3′
th1	Forward	5′- CTGGTTTCAAGATGGTGGA-3′
	Reverse	5′- TCAGAAGTTTGTTGGGAGG-3′
dat	Forward	5′-AGACTCCATCCCTCCCATAGC-3′
	Reverse	5′-CATCATTTACCCAGAAGCCATT-3′
drd1	Forward	5′-GGCTATTTCAACCAGGGTTCC-3′
	Reverse	5′-TGCGAATCACCAATGCTGT-3′
drd2a	Forward	5′-CTTCCCATCGGCGAAGC-3′
	Reverse	5′-CACCTTGTGGCGCAGC-3′
drd3b	Forward	5′- TCACTCGTTCAGACCCTCAT -3′
	Reverse	5′- ACGCTTTCCACGCACAT -3′
drd4	Forward	5′- CTGGTTTCAAGATGGTGGA-3′
	Reverse	5′- TCAGAAGTTTGTTGGGAGG-3′

high concentration group (32 mM). With the increase of INH concentration, the hatching of zebrafish was inhibited at 72−120 hpf (Fig. 1B). At 120 hpf, the hatchability in the 32 mM group was 54.67% as that of the control group.

Increasing the concentration of INH not only significantly inhibited the hatchability but also induced morphological abnormalities of zebrafish embryos (Fig. 1C and D). As shown in the figure, all organs in the normally developed zebrafish (control group) were clearly visible, pigments were evenly distributed, somites were clearly visible, and the swim bladder developed at 96 hpf. The morphology of larvae in the 2 mM group was not qualitatively different from that of larvae in the control group. Larvae in the 4 mM group at 120 hpf had a reduced swim bladder and an unclear distribution of dorsal pigments (Fig. 1E). Larvae in the 8 mM group did not have a swim bladder, the pigment boundary was blurred, and there was mild spinal curvature. Larvae in the 16 mM and 32 mM groups after continuous exposure for 120 h had malformation phenomena including obvious pericardial edema, spinal curvature, delayed yolk sac absorption, no swim bladder, and uneven distribution of pigmentation. INH significantly inhibited the normal development of zebrafish, and the percent of larvae with malformations increased in a concentration dependent manner (Fig. 1C). In normal 120 hpf zebrafish, the 3 primary regions of the brain can be discerned including the forebrain, midbrain, and hindbrain. The indentations defining the forebrain include one just posterior to the upper jaw/olfactory region and another above the eye. The midbrain junctions include the latter as well as a posterior junction centered above the otic capsule. Hindbrain brain includes the end of the midbrain to the lateral border. With the increase in INH concentrations, the boundarie of the forebrain, midbrain, and hindbrain were not clear, and obvious change in size and/or shape appeared (Fig. 1F).

3.3. Significant inhibition of the behavioral ability of zebrafish larvae by INH

After continuous INH exposure for 120 h, the behavior tracks of zebrafish larvae were recorded as shown in Fig. 2A. In the behavior tracks map, fast movement, medium movement and slow movement were indicated by red lines, green lines and black lines, respectively. Compared to the control group, the total movement distance of larvae significantly decreased (Fig. 2B) and the average movement speed significantly decreased (Fig. 2C) with the increase in INH concentration exposure. In the control group and the exposure groups, the movement conditions of larvae were mainly concentrated in medium-speed movements. With the increase in drug concentration, INH caused a significant decrease in the movement distances of larvae at low, medium, and high speeds, compared with that of larvae in the control group (Fig. 2D). Compared with that in the control group, the average moving speed of larvae in the 16 mM and 32 mM groups decreased significantly at all different time periods, and the movement ability was significantly inhibited (Fig. 2E).

3.4. Induction of apoptosis in the brain of zebrafish by INH

After AO staining, the apoptotic nuclei in zebrafish brain showed green yellow fluorescence under fluorescence microscope. At 120 hpf, a large number of apoptotic cells were found in the brain of zebrafish larvae at high concentrations group (16 mM and 32 mM), some apoptotic cells were also observed in the body of embryos, but no obvious apoptotic cells were observed in the control group (Fig. 3). These results indicated that INH induced apoptosis in the brain of zebrafish in a concentration-dependent manner.

3.5. Effects of INH on the development of vasculature in zebrafish larvae brain

Fli1 is an endothelial marker and the fli1 promoter is able to drive expression of GFP in all blood vessels. We used fli1:GFP transgenic zebrafish to investigate whether INH inhibited vascular development. As shown in Fig. 4, compared with those in the control group, neurovascular vessels in zebrafish in the 2 mM group did not change significantly. With the increase in concentration, INH significantly decreased the neurovascular vessels of zebrafish. In particular, the brain blood vessel structure in zebrafish was severely damaged at 120 hpf in the 16 mM and 32 mM groups, and brain blood vessel development was significantly suppressed (Fig. 4a'-f').

3.6. Induction of dopamine neuron injuries in zebrafish larvae by INH

It is known that dopamine neurons of zebrafish begin to form within 24 h of development, and all dopamine neurons have been fully developed within 4 days (Du et al., 2016). We observed the vmat2:GFP transgenic zebrafish developed for 5 days under a fluorescence microscope (Fig. 5A), and we can observe the dopamine neurons in the raphe nuclei of the midbrain, the nerve plexus of paraventricular organs, the pretectal neural cluster, the intermediate hypothalamus neural cluster, locus coeruleus, etc (Wen, L et al., 2008). We measured the length of dopamine neurons in the raphe nucleus region. As shown in Fig. 5C, dopamine neurons in zebrafish in the control group had clear morphology, had an ordered arrangement, and showed a linear shape. With the increase in the concentration of INH, dopamine neurons had a significantly shortened length and a reduced fluorescence intensity. Compared with the control group, the dopamine neuron lengths of zebrafish in the 16 and 32 mM groups were significantly decreased at 72 hpf, which were 74.8 ± 4.2% and 71.5 ± 4.7% of the control group, respectively (Fig. 5B).

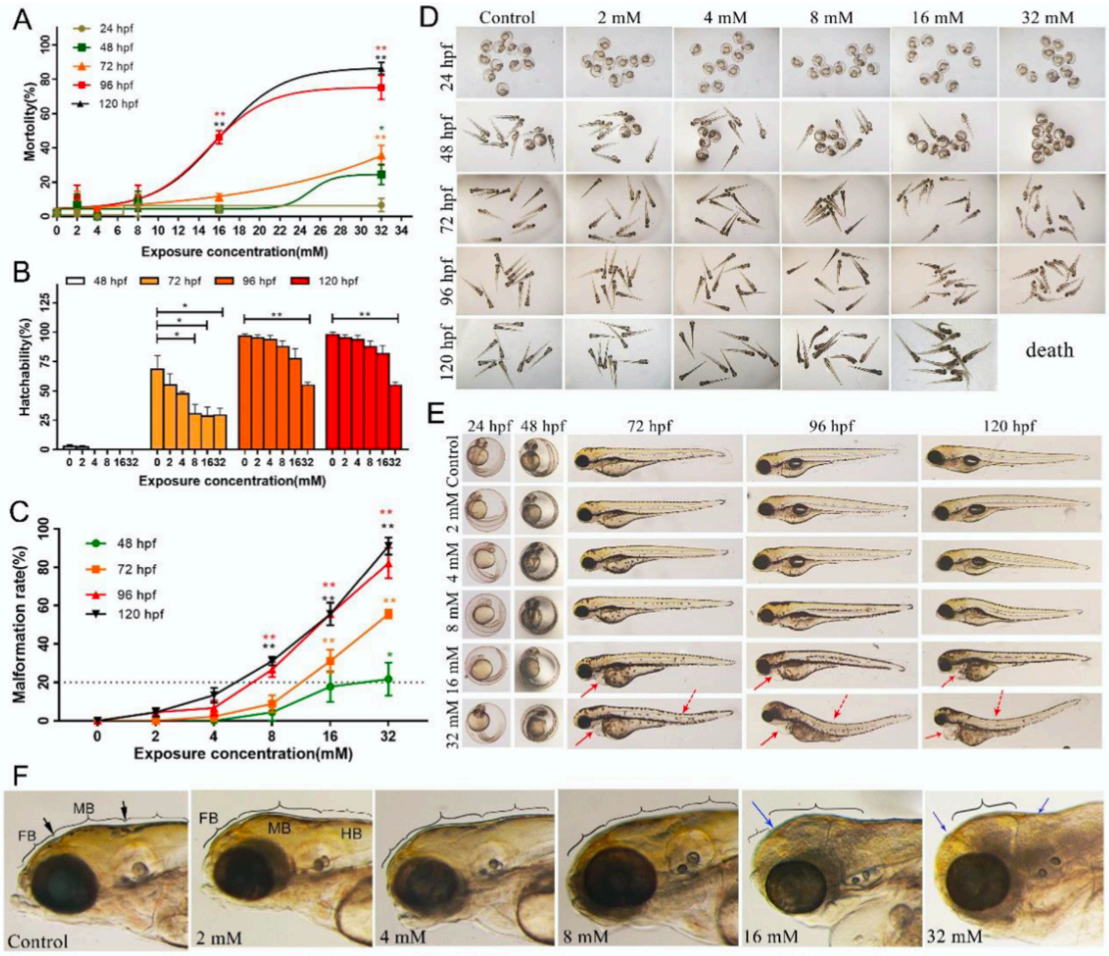

Fig. 1. Toxic effects of INH on the development of zebrafish embryos. (A) The percentage of zebrafish larvae deaths at 24–120 hpf. (B) Hatchability of zebrafish from 48 to 120 hpf. (C) Statistical analysis of the malformation of zebrafish from 48 to 120 hpf. (D) Overall developmental morphology of zebrafish from 24 to 120 hpf. (E) Developmental morphology of one single zebrafish from 24 to 120 hpf. Red solid arrows indicated pericardial edema, and red dotted arrows indicated spinal curvature. (F) Schematic diagram of the brain morphology of zebrafish exposed to different concentrations of INH at 120 hpf. Black arrow indicated the boundary between the normal midbrain and hindbrain, and blue arrows indicated that there is no clear connection of parts of the brain. Results are expressed as mean ± SEM. *$p < 0.05$ and **$p < 0.01$ vs the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. Effects of INH on the expression levels of genes associated with apoptotic pathways

Compared with the control group, INH can increase the mRNA expression levels of pro-apoptotic factors. *Caspase-3*, *caspase-8*, *caspase-9*, and *bax* in the IHN treatment groups were significantly up-regulated, and the mRNA expression levels of *bcl-2* in the 4, 8, and 16 mM groups were significantly decreased. Compared to that in the control group, *bcl-2/bax* in the 8 and 16 mM treatment groups significantly decreased (Fig. 6A).

3.8. Effects of INH on the expression of genes associated with the dopaminergic nervous system

Genes associated with the dopamine signaling pathway were detected using qPCR, including the gene expression levels of tyrosine hydroxylase (*th1*), dopamine transporter (*dat*), and dopamine receptors *drd1*, *drd2a*, *drd3*, and *drd4b* in zebrafish larvae. The results showed that the expression levels of *th*1 and *dat* significantly decreased with the increase in the INH concentration. Compared with that in the control group, the expression level of *drd2a* in the 16 mM group significantly decreased. The expression levels of *drd3* at all concentrations were all lower than that in the control group, with a significant decrease at 8 mM. *drd1* mRNA increased in 2 mM, 4 mM and then gradually decreased. When exposured with concentrations of INH (8 mM), *drd4b* mRNA increased ~2-fold, then decreased significantly following this peak. *drd1* mRNA increased in the 2 mM, 4 mM group and then gradually decreased in the 8 mM, 16 mM group. *drd4b* mRNA increased ~2-fold at 8 mM, then decreased significantly following this peak. (Fig. 6B).

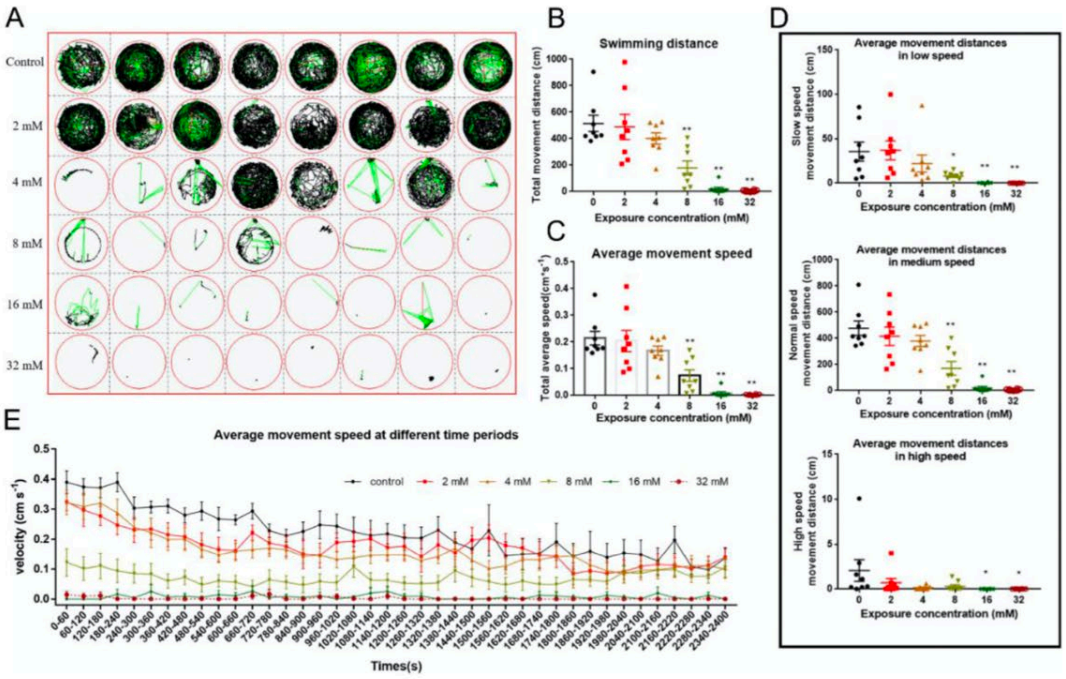

Fig. 2. Inhibitory effects of INH on movement behaviors of zebrafish larvae at 120 hpf. (A) Behavioral tracks of zebrafish larvae traced within 40 min. (B) Total swimming distances of zebrafish in all groups. (C) Average movement speed of zebrafish larvae in all groups. (D) Average movement distances at low, medium, or high speed. (E) Average movement speed of larvae for different time periods. Results are expressed as mean ± SEM (n = 8). *P < 0.05 and **P < 0.01 vs the control group.

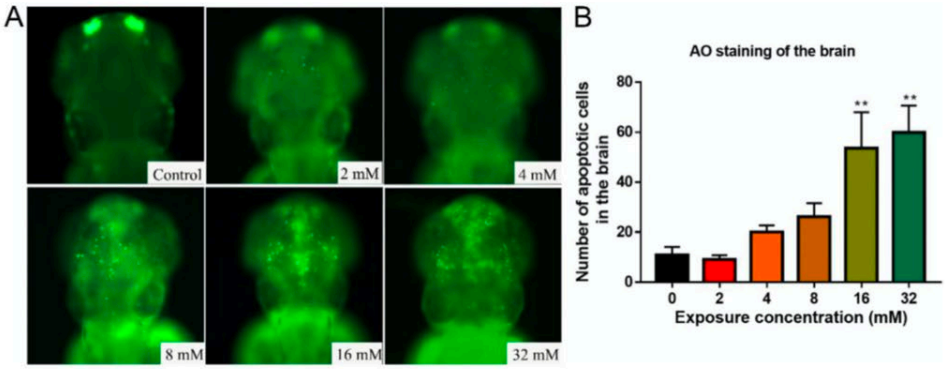

Fig. 3. INH-induced apoptosis in zebrafish brain. (A) After exposure with INH for 120 h, the zebrafish larvae were stained with acridine orange (AO). Schematic diagram of apoptotic cells in the brain of zebrafish larvae after AO staining. (B) Quantitative analysis of the number of apoptotic cells in zebrafish brain. Results are expressed as mean ± SEM (n = 8). *P < 0.05 and **P < 0.01 vs the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

This study investigated neurodevelopmental toxicity of INH in zebrafish embryos and the mechanism underlying its toxicity. INH produced significant neurodevelopmental toxicity in zebrafish embryos in a concentration dependent manner. INH caused the malformation of the larval brain, significantly decreased movement abilities, significantly reduced the length of dopamine neurons, inhibited brain blood vessel development, and induced apoptosis in brain. Apoptosis was also induced in the brain of 3 dpf larvae exposed to propofol.

Caspases are the core of apoptosis. *caspase-3* is the most

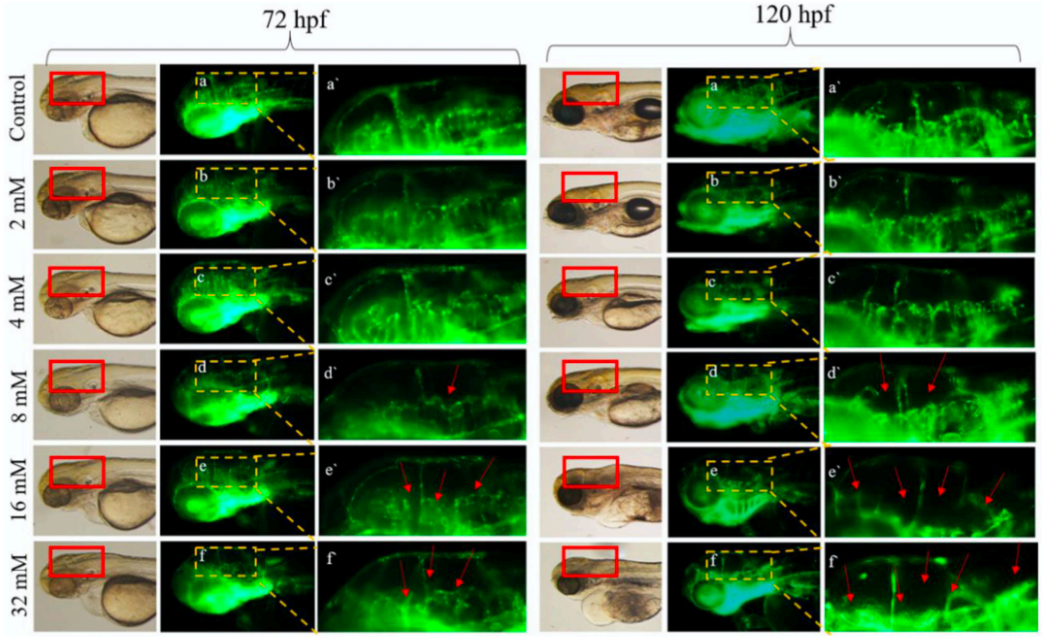

Fig. 4. Inhibition of brain blood vessel development in transgenic zebrafish Tg (fli1:GFP) larvae by INH at 72 hpf and 120 hpf. a-f Yellow dotted boxes indicate the brain blood vessel concentration areas. a'-f' Enlargement of brain blood vessels. Red arrows indicate the locations of blood vessel injury (n = 8). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

important terminal cleaving enzyme in the apoptosis process (Salvesen, 2002). A study by Yamashita et al. (2018) showed that enhanced *caspase-3* expression induced extensive apoptosis in the embryo of transgenic zebrafish. *caspase-8* belongs to initiator caspases and mediates death receptor-related apoptotic signals to further activate *procaspase-9*, while *caspase-9* initiates and mediates endogenous apoptosis (Greenjohn and Reed, 1998; Zou et al., 1997; Wyllie, 2010). The *bcl-2/bax* ratio determines whether cells will undergo apoptosis after stimulation by apoptotic signals; an increase in the *bcl-2/bax* ratio indicates apoptosis inhibition, and a decrease in the *bcl-2/bax* ratio indicates apoptosis promotion (Ji et al., 2013). Sharma et al. studied the nephrotoxicity of antituberculosis drugs on mice and showed that INH caused oxidative stress and mitochondrial damage, increased *bax* expression, and decreased *bcl-2* expression to induce apoptosis (Sharma et al., 2019). Guo et al. (2015) studied the neurodevelopmental toxicity of propofol on zebrafish, they found that propofol induced increased apoptotic cells in embryonic head, and the expressions of *caspase-3, -8* and *-9* were up-regulated, and apoptosis could be used as an evaluation index of neurotoxicity. In this study, with the increase in the concentration of INH, apoptotic cells in the brain of zebrafish significantly increased. The expression levels of *caspase-3, -8* and *-9* were significantly increased, while the expression of *bcl-2/bax* was significantly decreased by qPCR.

The development of blood vessels and neurons in the brain complements each other. It has been shown that zebrafish neurons can secrete exosomes containing microRNA 132 (mir-132) to regulate brain vascular formation (Xu et al., 2017). Studies have shown that increased neuronal vegfaa expression promotes angiogenesis and the formation of neurovascular networks (Wild et al., 2017; Madelaine et al., 2017). Due to the high metabolic rate of neurons, large amounts of nutrients are consumed. Blood vessels are channels for blood to transport oxygen and nutrients to the body. Vascular integrity helps to maintain homeostasis in the brain microenvironment (Alvarez et al., 2011; Ulrich et al., 2011); therefore, brain blood vessels are very important for the normal nerve function in the brain. Results from this study showed that INH severely inhibited the normal development of blood vessels in the brain of zebrafish and affected neurodevelopment in larvae.

As a major neurotransmitter, dopamine has important neural regulation functions. The vesicular monoamine transporter (*Vmat*) gene is localized on the cell membrane of dopamine neurons. The green fluorescence area in the brain of zebrafish in the transgenic (vmat2:GFP) is a dopamine neuron enrichment area; thus, the developmental process of dopamine neurons can be visualized and are easy to observe (Haque et al., 2015). Dopaminergic signaling pathway abnormalities usually cause neurodevelopmental toxicity in zebrafish. In order to explore the mechanism of INH inducing neurodevelopmental toxicity in zebrafish, the expression levels of the representative genes tyrosine hydroxylase (*th1*), dopamine transporter (*dat*), and dopamine receptor (*drd1, drd2a, drd3*, and *drd4b*) in the dopamine signaling pathway were detected. In the synthesis of dopamine, *Th* is one of the key rate-limiting enzymes (Ibáñez, 2008). *Th* limits the rate of conversion of phenylalanine to tyrosine and tyrosine to levodopa (L-DOPA) (Rink and Wullimann, 2002). Dopamine transporter (*dat*) is located at the dopaminergic endings and is a special dopaminergic neuron marker. Its function may be the same as human nigrostriatal or ventral tegmental dopaminergic neurons (Xi et al., 2011). *Dat* plays a critical role in the termination of neurotransmitter release and the maintenance of neurotransmitter balance in the body. Zhao et al. (2012) showed that zebrafish with reduced *th1* and *dat* expression exhibited severe

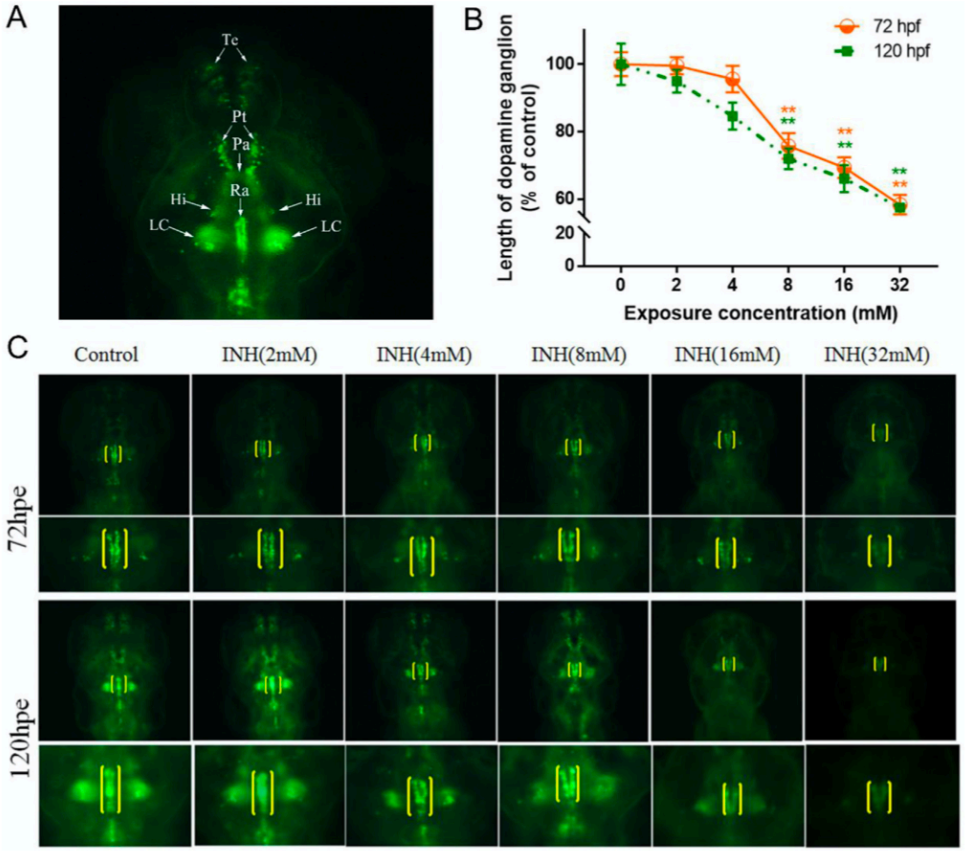

Fig. 5. Induction of dopamine neuron injury in transgenic zebrafish Tg (vmat2:GFP) larvae by INH at 72 hpf and 120 hpf. (A) Dopamine neurons distribution of 120 hpf vmat2:GFP transgenic zebrafish. (B) Statistical analysis of the length of dopamine neurons in the raphe nucleus. (C) Representative fluorescent microscopy images of vmat2:GFP zebrafish were exposed to INH and the effect at 72 hpf and 120 hpf. Te: telencephalic neurons; Pt: pretectal neural cluster; Hi: intermediate hypothalamus neural cluster; Ra: raphe nuclei; LC: locus coeruleus; Pa: neural cluster of paraventricular organ. White arrowheads in panels A: GFP-positive neurons in midbrain. Yellow brackets in panels C: Dopamine neurons in raphe nucleus. Results are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$ vs the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

movement disorders (bradykinesia). In this study, we detected the gene expression level of dopamine signaling pathway, and found that the expression levels of *th* and *dat* genes decreased with the increase of INH concentration. According to the statistics of 40 min in Fig. 2E, zebrafish with high concentration (16 mM, 32 mM) showed poor response to new environment, and the average moving speed was almost 0. Zebrafish larves in the high-concentration INH group significantly reduced their exercise capacity at 120 hpf. Compared with the control group, the low concentration group (such as 2 mm, 4 mm INH) had no significant effect on the total movement distance. According to the total movement distance of high, medium and low speed, we found that exposure to INH reduced the middle and high speed movement of zebrafish, and increased the low/stationary motion. The results indicated that INH interferes with the dopamine signaling pathway and affects the normal transmission of neurotransmitters in the body, causing significant neurotoxicity to zebrafish.

Based on biochemical and pharmacological properties, dopamine receptors can be classified into D1-like receptors and D2-like receptors. Dopamine D1 receptor (D1R) plays an important role in autonomous movement, memory, and attention, especially reward-related learning (Beninger and Miller, 1998). Dopamine can regulate neuronal activities and the working memory process in the prefrontal cortex (PFC) through D1 receptors (Li et al., 2018). It has been reported that D2 receptor knockout mice exhibit impaired motor function, including reduced movement and incoordination (Shontz et al., 2018). Nguyen et al. studied the neurotoxicity of methamphetamine and tested dopaminergic parameters. Their results suggest that dopamine D1 and D2 receptors simultaneously mediate MPA-induced dopaminergic neurodegeneration in mice via oxidative burdens, microgliosis, and pro-apoptosis (Nguyen et al., 2019). We detected the expression levels of dopamine receptor genes and showed that the mRNA expression levels of *drd1*, *drd2a*, and *drd3* all significantly decreased, affecting the normal

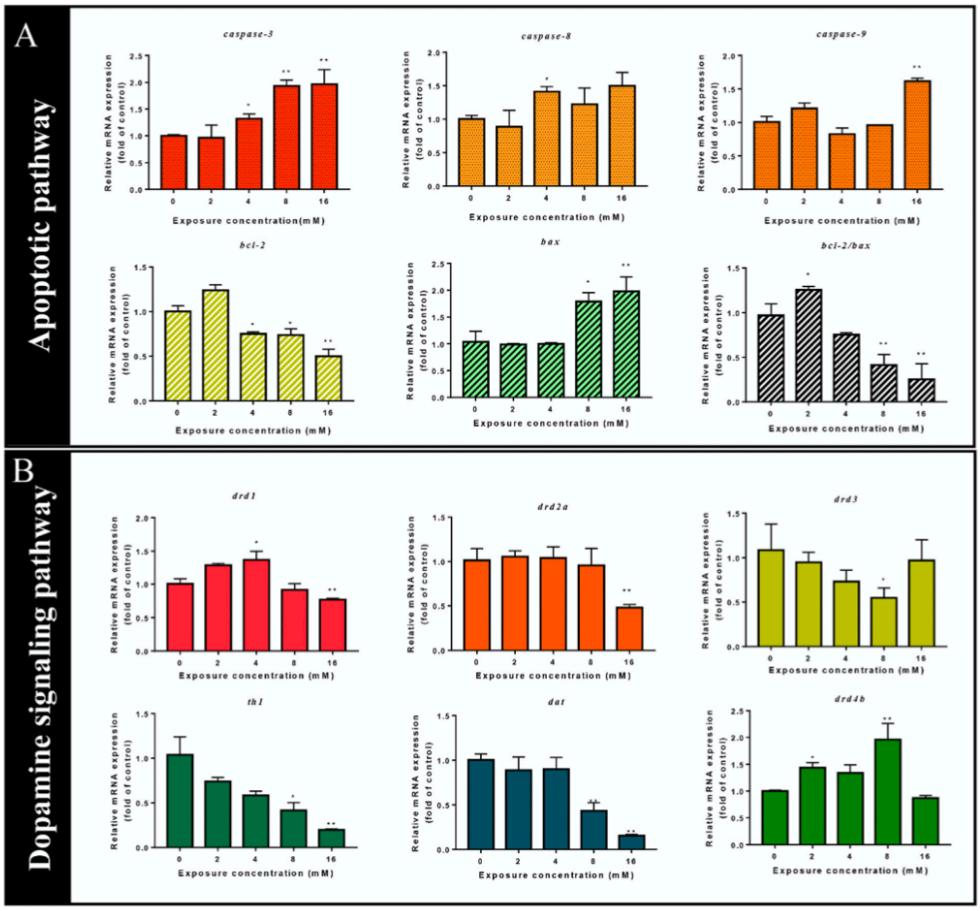

Fig. 6. Effects of INH on the expression of apoptosis- and dopamine-related gene in zebrafish larvae at 120 hpf. (A) The relative mRNA expression levels of apoptosis-related genes *caspase-3*, *caspase-8*, *caspase-9*, *bax*, and *bcl-2* after 120 hpf of INH treatment. (B) The relative mRNA expression levels of dopamine-related *drd1*, *drd2a*, *drd3*, *drd4b*, *th1*, and *dat* after 120 hpf of INH treatment. (n = 3). $*p < 0.05$, $**p < 0.01$ vs the control group.

transduction of dopamine neuron signals and inducing neurotoxicity. The expression level of *drd4b* mRNA first increased and then decreased from the increase in the concentration of INH. It is speculated that changes in the mRNA levels of *drd4b* might be a form of negative feedback regulation in the body. These results suggest that dopamine signaling pathway may be involved in INH-induced neurodevelopmental toxicity in zebrafish.

5. Conclusion

INH had obvious developmental toxicity to zebrafish embryos in a concentration-dependent manner. INH caused toxicity to the neural development of zebrafish, significantly reduced behavioral ability, altered vasculature of the brain, increased the expression level of apoptotic genes, and reduced the expression level of dopaminergic genes. The results of this study can provide a theoretical basis for the detection and treatment of clinical and adverse reactions of INH.

Credit author statement

Li Liu: Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition. Fang-yan Wu: Data curation, Writing – original draft, Funding acquisition. Chengyue Zhu: Data curation, Investigation, Validation, Software, Formal analysis. Hong-yuan Zou: Resources, Data curation, Visualization, Project administration, Funding acquisition. Rui-qi Kong: Investigation, Validation, Software, Data curation. Yu-kui Ma: Validation, Software. Dan Su: Investigation, Validation, Software, Formal analysis, Funding acquisition. Guo-qiang Song: Methodology, Resources. Yun Zhang: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Project

administration, Funding acquisition. Ke-chun Liu: Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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