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Saikosaponin B1/D alleviate dextran sulfate sodium-induced colitis via regulating the NRF2/HO-1 pathway to inhibit the ferroptosis in zebrafish

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Abstract:

Background: Inflammatory bowel disease (IBD) is a chronic and recurrent gastrointestinal inflammation, the pathophysiological mechanisms of that is not fully understood, and the current conventional treatment drugs are often associated with serious side effects. Saikosaponins(SSs) are the main active component of Bupleurum chinense DC. (BC). Saikosaponin A has been reported to have a positive effect on the remission of DSS induced colitis.

Aim: However, the studies on the mechanism and safety of SSs are still limited.

Materials and methods: We established zebrafish colitis model induced by sodium glucan sulfate (DSS) and gave intervention treatment with different saikosaponins.

Results : It was found that saikosaponin B1 (SSB1) and saikosaponin D (SSD) had the most prominent inhibitory ability on neutrophils infiltration in the larval intestine among the 6 saikosaponin monomers. In this study, we explored the effect and mechanism of SSB1 and SSD on DSS induced colitis in zebrafish. The results showed that both SSB1 and SSD could reduce the histological injury, tissue inflammatory cytokines and ROS expression in zebrafish. Moreover, we observed that both SSB1 and SSD significantly

inhibited ferroptosis in DSS stimulated zebrafish colitis. In **Conclusion:** conclusion, our results suggested that Saikosaponin B1/D play a protective role in inhibiting ferroptosis by up-regulating NRF2/HO-1 pathway.

Keywords: inflammatory bowel disease; saikosaponins; ferroptosis; NRF2/HO-1 pathway; zebrafish

Abbreviations

IBD : Inflammatory bowel disease	UC : Ulcerative colitis
CD : Crohn's disease	MDA : Malondialdehyde
PTGS2 : Prostaglandin peroxidase synthase 2	GSH : Glutathione
Nrf2Nu: Clear factor erythroid 2-related factor 2	ROS : Reactive oxygen species
HO-1 : Heme oxygenase-1	TCM: Traditional Chinese medicine
BC: Bupleurum chinense DC.	SSs: Saikosaponins
SSA: Saikosaponin A	SSB2: Saikosaponin B2
SSC: Saikosaponin C	SSD: Saikosaponin D
SSB1:Saikosaponin B1	SSF: Saikosaponin F
DSS: Dextran sodium sulfate sulfoxide	DMSO: Dimethyl
STC: Spontaneous tail contraction	

1. Introduction

Inflammatory bowel disease (IBD) is characterized by bloody diarrhea, abdominal pain fecal urgency and tenesmus, that includes two forms: ulcerative colitis (UC) and Crohn's disease (CD) [1]. IBD has developed into a global disease with rising prevalence all over the world, and its chronic recurrence and unpredictability potentially lead to economic burden of medical care, poor well-being and quality of life on patients. IBD is generally thought to be associated with interactions between host genetics, environmental factors, gut microbiota, and immune responses;

however, the explicit mechanism of disease pathogenesis remain unknown [2]. Recent reports suggest that ferroptosis is involved in the development and progression of IBD [3].

Ferroptosis is an iron-dependent form of nonapoptotic cell death caused by lipid peroxidation [4]. Iron metabolism and lipid peroxidation are considered to be key factors in ferroptosis. Ferritin is an iron-storage protein composed of two subunits: a heavy chain (FTH1) that possesses ferrous oxidase activity and converts Fe^{2+} to Fe^{3+} for safe storage, and a light chain (FTL) that lacks this activity [5]. Iron metabolism plays an important role in reducing free iron levels by storing excess cellular iron. In addition, increased expression of malondialdehyde (MDA) and prostaglandin peroxidase synthase 2 (PTGS2) are key features of ferroptosis [6]. Glutathione (GSH) is a powerful scavenger of lipid peroxidation products, and impaired GSH metabolism is one of the main mechanisms of ferroptosis [4,7]. There has been increasing evidences suggest that ferroptosis is associated with the pathogenesis of IBD, and suppressing ferroptosis could significantly ameliorate pathological damage of colitis in mice [8], [9]. These findings suggest that ferroptosis plays an important role in the treatment of UC. Moreover, it has been reported that Nrf2/HO-1 pathway is one of the regulatory mechanisms of ferroptosis [10]. Nuclear factor erythroid 2-related factor 2(Nrf2) is not only involved in preventing lipid peroxidation and the accumulation of free iron but also serves as a crucial transcription factor for antioxidant stress [11]. Excessive accumulation of reactive oxygen species (ROS) in cells translocates Nrf2 into the nucleus, activates heme oxygenase-1 (HO-1), and inhibits cell ferroptosis [12]. Therefore, it is very essential to find other drugs that can prevent ferroptosis to make a therapeutic

strategy for UC.

At present, the current therapies for UC mainly include aminosalicylates, corticosteroids, immunosuppressants and biological, but these therapies are usually accompanied by serious side effects [13]. Therefore, it is indispensable to explore novel approaches to attenuate UC effectively. In recent years, natural medicines have provided new therapeutic alternatives. A large number of clinical trials and modern experimental studies also show that traditional Chinese medicine (TCM) prescriptions and plant secondary metabolites may have great potential in the treatment of UC [14,15]. Our group's previous study found that Sinisan was effective in the treatment of UC [16]. We further conducted an in-depth study on *Bupleurum chinense* DC. (BC), which plays a major role in the compound. BC is a widely used Chinese herb with a long history of being used for inflammatory diseases, infectious diseases and liver diseases[17]. Saikosaponins (SSs) are considered as the main active components of BC, which has been confirmed to have anti-inflammatory [18], antibacterial [19], antiviral [20], anti-tumor [21], anti-epileptic [22], sedative and analgesic [23], liver protection [24], oxidative stress [25]and immune regulation [26] pharmacological effects. There were lots of anti-inflammatory studies on SSs. Saikosaponin A (SSA) protected against DSS-induced colitis through dose-dependently inhibiting expression of inflammatory cytokines via activation of LXR α [27]. Saikosaponin B2 (SSB2), saikosaponin C (SSC) and saikosaponin D (SSD) also showed effective anti-inflammatory activity [28-30]. However, there has been no research systematically conducted to determine the effectiveness of SSs including SSA, saikosaponin B1(SSB1), SSB2, SSC, SSD and saikosaponin F (SSF) on ameliorating UC. It is worth advocating

the use of high-throughput and low-cost experimental animal models to screen a large number of drugs. Zebrafish has become one of the most common animal models in molecular biology because of its advantages such as high throughput and easy operation. Moreover, the zebrafish gastrointestinal system is functionally and genetically homologous to the mammalian gastrointestinal system, suggesting that it is a suitable animal model. Chemical dextran sodium sulfate (DSS) -induced intestinal epithelial injury mimics a key feature of IBD pathology. DSS induced IBD zebrafish models successfully demonstrated neutrophil infiltration at 0.5% DSS concentration[31]. Acute mortality and levels of inflammation in colitis were criteria for assessing the effectiveness of the drugs.

The aim of this study was to evaluate the effects of SSA, SSB1, SSB2, SSC, SSD and SSF on intestinal inflammation, and to explore their molecular mechanisms and toxic effects in the zebrafish models.

2. Materials and Methods

2.1. Chemical and reagents

Saikosaponin A (SSA, A14GB145174), saikosaponin B1 (SSB1, A22GB146239), saikosaponin B2 (SSB2, M05GB140794), saikosaponin C (SSC, M12O11S126866), saikosaponin D (SSD, O11GB163264) and saikosaponin F (SSF, M28O10S101347) were purchased from Yuanye Bio-Technology (Shanghai). The purity of these reference saikosaponins was greater than or equal to 98%. Dextran sulfate sodium salt (DSS, M.W 40000, C14161866) was obtained from Macklin (Shanghai, China). Mesalazine (153463) was purchased from MedChemExpress. SYBR qPCR Master Mix (Q711-02) and the BCA protein quantification kit-box2 (E112-01) were purchased from Vazyme (Nanjing, China). FastKing gDNA

Dispelling RT SuperMix (#X0402) was obtained from TIANGEN BIOTECH (Beijing). Malondialdehyde (MDA) assay kit (20230227) reduced Glutathione (GSH) Content Assay Kit (20230309) and tissue iron (Fe) assay kit (20230306) were provided by Nanjing Jiancheng Bioengineering.

2.2. Zebrafish maintenance and Drug Treatment

Wild-type (WT) AB and Transgenic neutrophilic fluorescent zebrafish Tg (mpx:EGFP^{il14}) were provided by LONGSEEK of Guangdong Longsee Biomedical Corporation. Adult zebrafish were maintained under a 14/10 h light/dark cycle at 28 ± 0.5 °C, and fed brine shrimp twice per day regularly. One day before the experiment, healthy adult zebrafish with reproductive ability were placed in incubator with a 1:1 or 1:2 male to female ratio, separated by a partition. The partitions were pulled out at 9:00 the next morning, and the fertilized naturally zebrafish embryos were collected from the incubators and cultured in E3 water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) at 11:00. The E3 water for cultivating embryos was changed twice every day. Then the 3 days post-fertilization (dpf) larvae were randomly separated into nine groups, including control group, DSS group, DSS + SSB1 (1.5 μM), DSS + SSB1 (3 μM), DSS + SSB1 (6 μM); DSS + SSD (0.075 μM), DSS + SSD (0.15 μM), DSS + SSD (0.3 μM) and DSS + MSLZ (50 ug/mL) group. The DSS-induced intestine injury model was adapted from Oehlers et al [31]. The zebrafish model with intestinal dysfunction was established with 0.5% DSS for 2 days at 3 dpf, and treated with SSs for 2 days at 5 dpf. Simultaneously, mesalazine, an anti-inflammatory drug commonly prescribed for IBD treatment, was used as a positive group. All analyses were performed at 7 dpf unless otherwise stated.

2.3. Safe assessment of saikosaponins

The healthy zebrafish embryos (WT; AB strain) were selected and divided randomly in 6 well plates (20 larvae per well) at 2 hours post-fertilization (hpf). The SSs were weighed and dissolved in dimethyl sulfoxide (DMSO). Different concentrations SSs were prepared with E3 water. Next, the zebrafish embryos were treated with different concentrations (0, 1.562, 3.125, 6.25, 12.5, 25 and 50 μ M) of SSs, each group was repeated with 3 wells, and the drug was administered for 120 h continuously. After administration, the 6 well plates were cultured in an incubator at 28.5 °C. The mortality rate, hatching rate and malformation rate of zebrafish embryos were observed and counted every day. When embryos appeared yolk sac edema, pericardial edema, pericardial hemorrhage, tail bending, spine curvature and death, etc., the concentrations of drugs were considered toxic. Hence, we obtained three safe concentrations: low, medium and high for each candidate SSs.

2.4. High-throughput screening and data analysis

The safe concentrations of 6 SSs were obtained by drug exposure treatment in the previous stage. The 3 dpf zebrafish larvae (Tg (mpx:EGFP^{il14})) were placed in 6 well plates and randomly divided into control group, DSS group, DSS+SSs (low concentration), DSS+SSs (medium concentration), DSS+SSs (high concentration) (N=12). Except the control group, all groups were exposed to a total volume of 5 ml 0.5% DSS for 2 days, and the DSS was dissolved by E3 water. After 2 days of DSS exposure, the 5 dpf larvae were washed with E3 water and placed into SSs (three safe concentrations) or 50 μ g/mL mesalazine for 48 hours, and the DSS group treated with E3 water. The 7 dpf larvae were anesthetized with 0.02% tricaine for live imaging by fluorescence

microscopy. The degree of inflammation was indicated by the number of neutrophils recruited in the intestine. The neutrophils in the larval intestine were counted by manual counting. The obvious reduction of neutrophils infiltration was defined as a hit. Larval images were taken using a Nexcope NIB900.

2.5. Histological Analysis

2.5.1. Hematoxylin and Eosin Staining

The 7 dpf zebrafish larvae were fixed overnight in 4% paraformaldehyde at 4 °C and then progressively dehydrated in ethanol. The larvae were embedded in paraffin, cut into 3 µm transverse and longitudinal sections and then stained with hematoxylin-eosin.

2.5.2. Alcian Blue Staining

DSS-stimulated intestinal damage in larvae leads to accumulation of acid mucin. [32]. Intestinal goblet cells secrete mucin and form mucous layer in homeostasis, which plays an important role in maintaining intestinal immune homeostasis [33]. In order to observe the changes of intestinal goblet cells, alcian blue staining of acid mucin was used. The larvae were fixed in 4% paraformaldehyde at room temperature for 2 h, and then dehydrated and embedded. The sections were stained with Alcian Blue according to standard protocols

2.6. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 30 zebrafish larvae with RNA Easy Fast Tissue/Cell Kit according to the reagent vendor's instructions. The complementary DNA (cDNA) was synthesized with FastKing gDNA Dispelling RT SuperMix and the system with the following conditions: 42

°C for 15 min and 95 °C for 3 min. Real-time PCR assay was performed with the SYBR green qPCR Master and the conditions were as follows: 3 min at 95 °C for 1 cycle, 10 s at 95 °C and 30 s at 60 °C for 40 cycles. β -actin was served as the internal reference. The sequences of primers used for RT-PCR are listed in the Table 1. The relative expression of the target genes was measured using the $2^{-\Delta\Delta CT}$ methodology.

Table 1
Quantitative PCR primer sequences.

Name	Primer	Sequence
β - actin	Forward	5'CCATCTATGAGGGTTACGC 3'
	Reverse	5'GACAATTTCTCTTTTCGGCT 3'
IL-6	Forward	5'TCAACTTCTCCAGCGTGATG3'
	Reverse	5'TCTTTCCCTCTTTTCCTCCTG3'
IL-8	Forward	5'GTCGCTGCATTGAAACAGAA3'
	Reverse	5'CTTAACCCATGGAGCAGAGG3'
MMP9	Forward	5'CTTCAAGGACGGGCGCTACT 3'
	Reverse	5'GAGGTGGTCCTCAAAGGCAG 3'
IL-1 β	Forward	5'CTCAGCCTGTGTGTTTGGGA 3'
	Reverse	5'GGGACATTTGACGGACTCG 3'
TNF- α	Forward	5'GCTGGATCTTCAAAGTCGGGTGTA 3'
	Reverse	5'TGTGAGTCTCAGCACACTTCCATC 3'
Nrf2	Forward	5'TCGGGTTTGTCCCTAGATG 3'
	Reverse	5'AGGTTTGGAGTGTCCGCTA 3'
HO-1	Forward	5'ATGCCCTTGTTTCCAGTCAGC 3'

	Reverse	5'GGACTTGGAGCACTTCTTCGG3'
PTGS2	Forward	5'TGGATCTTTCCTGGGTGAAGG 3'
	Reverse	5'GAAGCTCAGGGGTAGTGCAG3'
FTH1	Forward	5'-CAGGACGTGAAGAAACCAGAGAAGG 3'
	Reverse	5'-ATGTGAGGGTCGTTGTGTTGAGATG 3'
FTL	Forward	5'ATGCAGCCACTCTGGGTAAA 3'
	Reverse	5'AATCCATCTTGCTTGTTTTAGGGC3'

2.7. ROS level analysis

DCFH-DA probes were used to measure the ROS levels in zebrafish larvae tissues. The 7 dpf larvae (WT; AB strain) after drugs treatment were placed in 96-well plates and incubated for 30 min with 5 μ M DCFH-DA staining in the dark. Then, the zebrafish larvae were rinsed with E3 water for 3-5 times, and anesthetized using 0.02% tricaine. The lateral view images were taken using a fluorescence stereomicroscope. The content of ROS was expressed in fluorescence intensity, which was quantified using image J software.

2.8. Reduced GSH assay

The 30 larvae were placed in a 1.5 mL centrifuge tube with 200 μ L normal saline, and grinded with a grinding gun. Then, centrifuged at 12,000 x g for 2 min, the supernatant was collected and transferred to another clean tube. The supernatant was used for this experiment. The GSH content was assessed using a reduced Glutathione (GSH) Content

Assay Kit according to the manufacturer's instructions. The absorbance was measured at 405 nm with an enzyme marker.

2.9. Malondialdehyde (MDA) assay

MDA content in the supernatant was measured using a malondialdehyde kit. A negative control must be set up for each measurement sample according to the manufacturer's instructions. After each tube was added to reagents in turn, which was tied with plastic wrap. And then the tubes were immersed in water at 95°C for 80 minutes. After that, the absorbance of each tube was measured at 532 nm.

2.10. Iron assay

Zebrafish larvae tissue proteins were prepared as stated in the iron assay kit. The total iron in the supernatant of larvae was measured with a tissue iron (Fe) assay kit. According to the manufacturer's instructions, each tube was sequentially added with reagents, mixed, boiled in water for 5 minutes and cooled by running water. Then, centrifuged at 12,000 x g for 2 min, collected supernatant and transferred to a clean tube. The absorbance was measured at 520 nm. The protein concentration (C_{prot}) was measured by plotting a standard curve using the BCA Protein Assay Kit.

2.11. Measurement of toxicity endpoints

The effects of SSB1 and SSD on zebrafish development were evaluated, including mortality, hatching rate, body length, yolk sac area, heart rates

and behavior analysis. Specifically, the 2 hpf embryos were cultured in 6-well plates and exposed to different concentrations of SSB1 and SSD. And the mortality and hatching rates were recorded at 24, 48, 72, and 96 hpf. The heart beat was assessed by a counter. The embryos were placed to a glass slide at 48 hpf and videotaped for 10 seconds under a stereomicroscope. And the results were showed an average heart rate per second. Lateral images of each zebrafish were collected at 96 hpf using fluorescence microscope, and the body length and yolk sac area of larvae were measured using Image J. Behavioral tests were conducted to determine whether SSB1 and SSD exposure affected zebrafish motor behavior. Two types of motor behavior tests were performed, including embryonic spontaneous tail contraction (STC) and touch-induced movement. The STC experiments were performed as follows: the embryos with drug treatment for 24 hours were placed in 6-well plates and the alternately bending tail or curling up of each embryo were videotaped for 30 seconds by stereomicroscopy. The test of touch-induced movement was to induce stress response by gently touching the back and tail of zebrafish with a blunt after exposing to SSB1 or SSD 36-72 h. The average escape times of larvae were recorded, and the touching times of each zebrafish larvae were 20 times.

2.12. Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0.

Student's t-test for unpaired results was used to evaluate differences between two groups. One-way ANOVA was adopted for detecting the statistical significance among different dose treatments. The P values of less than 0.05 were considered statistically significant. Mean $\pm$ the standard error of the mean (SEM) is used to display data.

3. Results

3.1. Safe assessment of saikosaponins

In order to obtain the safe concentration of SSs on zebrafish Larvae, the acute toxicity tests were conducted. The results were as follows: three safe concentrations of 1.5 μ M, 3 μ M and 6 μ M for SSA and SSB1; 3.125 μ M, 6.25 μ M and 12.5 μ M for SSB2, SSC and SSF, respectively; and 0.075 μ M, 0.15 μ M and 0.3 μ M for SSD.

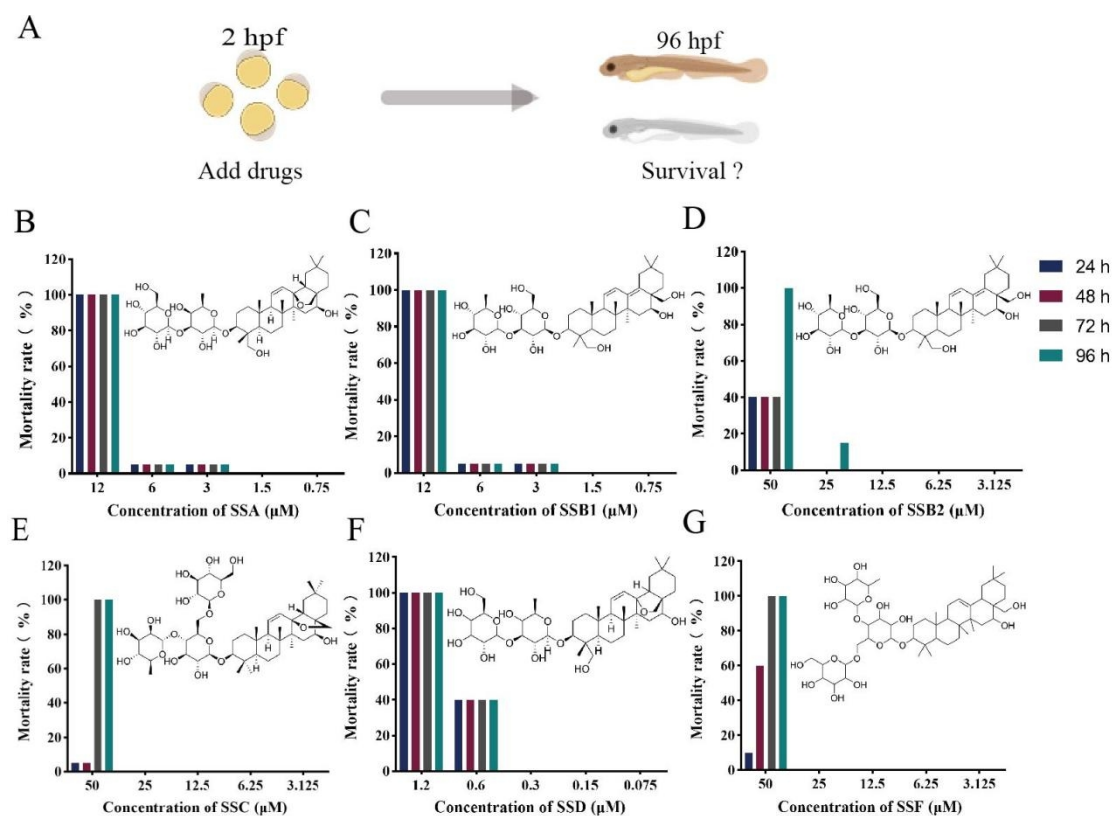


Figure. 1 Acute toxicity of SSs and chemical structures of SSs. (A) The schematic diagram describes the statistical method for mortality of zebrafish larvae under

the administration of SSs. Mortality of zebrafish larvae after 96 h exposure to SSA and the chemical structure of SSA (B), SSB1 (C), SSB2 (D), SSC (E), SSD (F), SSF (G), expressed as mean $\pm$ SEM, N=20.

3.2. SSB1 and SSD reduced inflammation in DSS-exposed larvae

Further anti-inflammatory screening was conducted based on results from the previous toxicity trials. Neutrophils recruitment in intestinal of Tg (mpx:EGFP)^{il14} larvae was assessed by stereopic fluorescence microscopy at 7 dpf. The number of intestinal neutrophils was statistically significant in the 0.5% DSS group versus the control group, representing the successful construction of colitis model. Our results showed that the accumulation of neutrophils was significantly inhibited by treatment of SSA, SSB1, SSB2 and SSD, but SSC and SSF had no significant effect on alleviating neutrophils mobilization in the gut of larvae. Based on the inhibitory effect on neutrophil recruitment and the concentration of SSs, we decided to investigate SSB1 and SSD in depth.

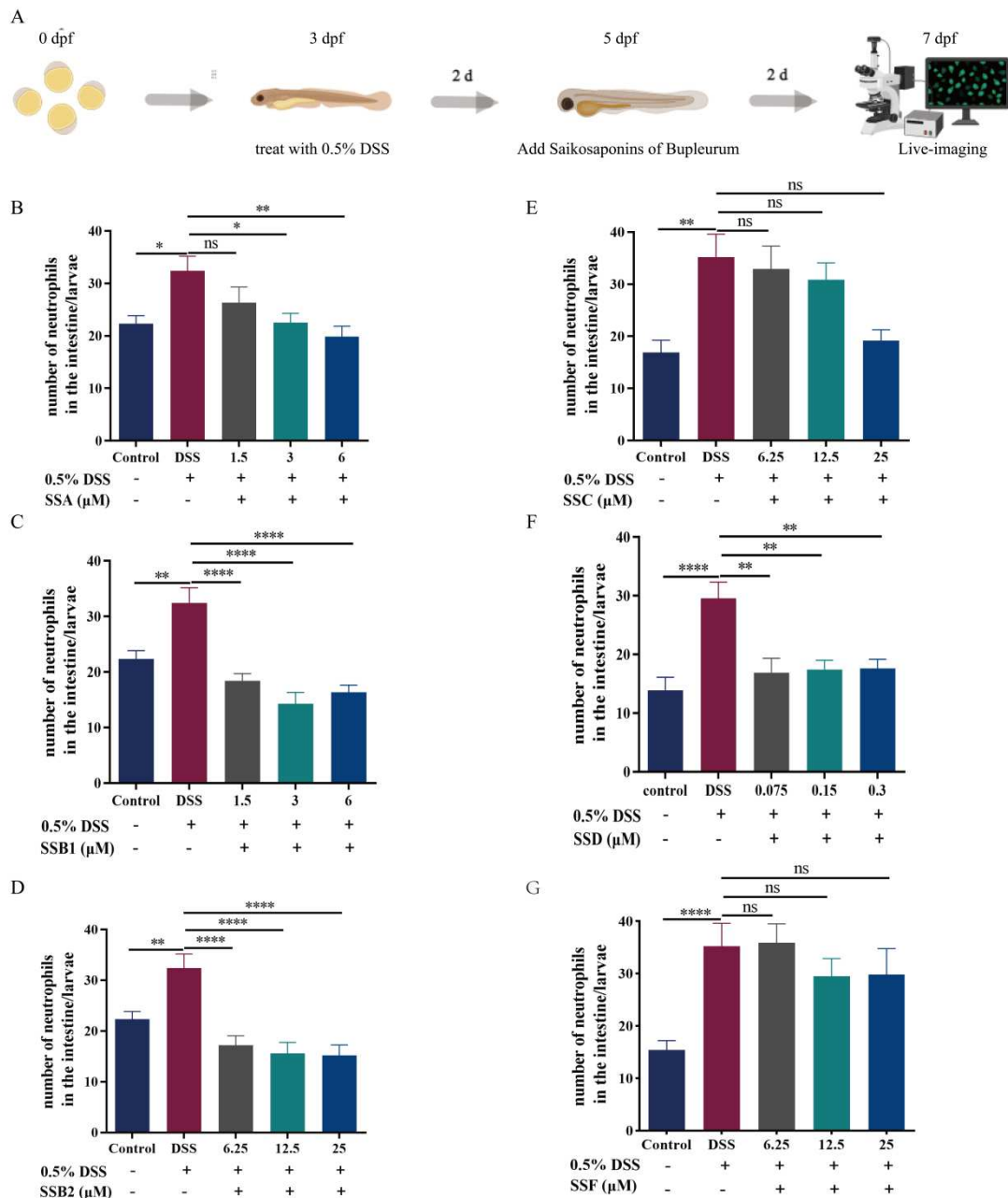


Figure. 2 Effects of 6 SSs on intestinal inflammation (A) Schematic diagram describes screening methods for SSs to suppress neutrophil inflammation. Tg (mpx:EGFP)ⁱ¹¹⁴ larvae exposed to 0.5% DSS for 2 days and treated with SSA (B), SSB 1 (C), SSB 2 (D), SSC (E), SSD (F), SSF (G) for 2 days, and the number of neutrophils in the intestine of per larvae. Mesalazine (MSLZ) was the positive group. The number of intestinal neutrophils was counted by representative live imaging figure S1 and the intestinal region is indicated by the white dashed line. Data were presented as mean $\pm$ SEM, $N \geq 12$. The values represented by different letter columns were significantly different ($P < 0.05$, one-way ANOVA).

3.3. SSB1 and SSD attenuated histological damage induced by DSS in zebrafish

Intestinal transverse and longitudinal sections of 7dpf zebrafish larvae were stained using hematoxylin and eosin (H&E) to assess intestinal villus structure, integrity of intestinal epithelial cells integrity, the presence of mature cup cells, and intestinal cross-sectional cavity area. There was a significant change in intestinal morphology after DSS challenge. We observed that the intestinal walls of the DSS-induced zebrafish larvae became thinner, the intestinal lumen area became larger, the intestinal muscle layer became thinner (Fig. 3.A), the villi of the intestines became shorter. intestinal epithelium shedding was serious and the cupped cells increased. (Fig. 3.B). The Alcian Blue staining results showed that the accumulation of intestinal acid mucin and proliferation of mature goblet cell in DSS group (Figure 3.C). However, the SSB1 (6 μ M) and SSD (0.3 μ M) administration obviously reversed the degree of intestinal epithelium damage after DSS challenge.

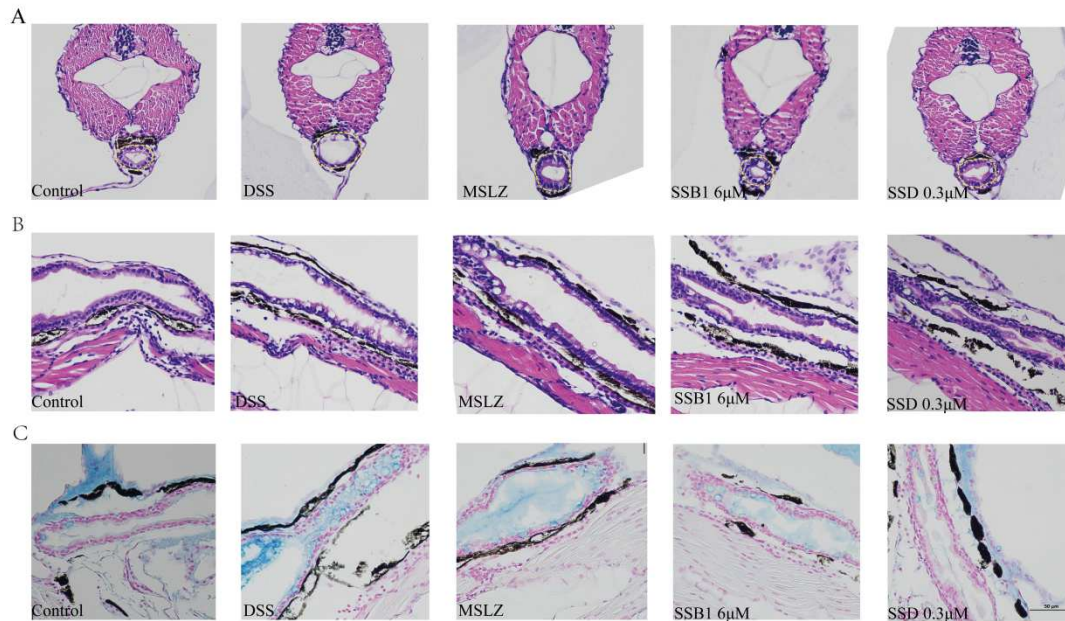


Figure. 3 SSB1 and SSD protect intestinal epithelium. (A) Hematoxylin and eosin (H&E) staining on transverse section of zebrafish larval intestine. (B) Hematoxylin and eosin (H&E) staining on longitudinal section of zebrafish larval intestine. (C) Alcian Blue staining on longitudinal section of zebrafish larval intestine, scale bars, 50 μm.

3.4. SSB1 and SSD decreased inflammatory cytokine expression in zebrafish with DSS-induced colitis

We further examined the effects of SSB1 and SSD on DSS-induced pro-inflammatory cytokines expression by RT-qPCR. As shown in Fig.4, the mRNA expressions of MMP9, IL-6, IL-8, IL-1 β and TNF- α were significantly increased in DSS-treated larvae (Fig.4). The SSB1 and SSD significantly reversed the up-regulated expression of all candidate inflammatory cytokines compared with the DSS group.

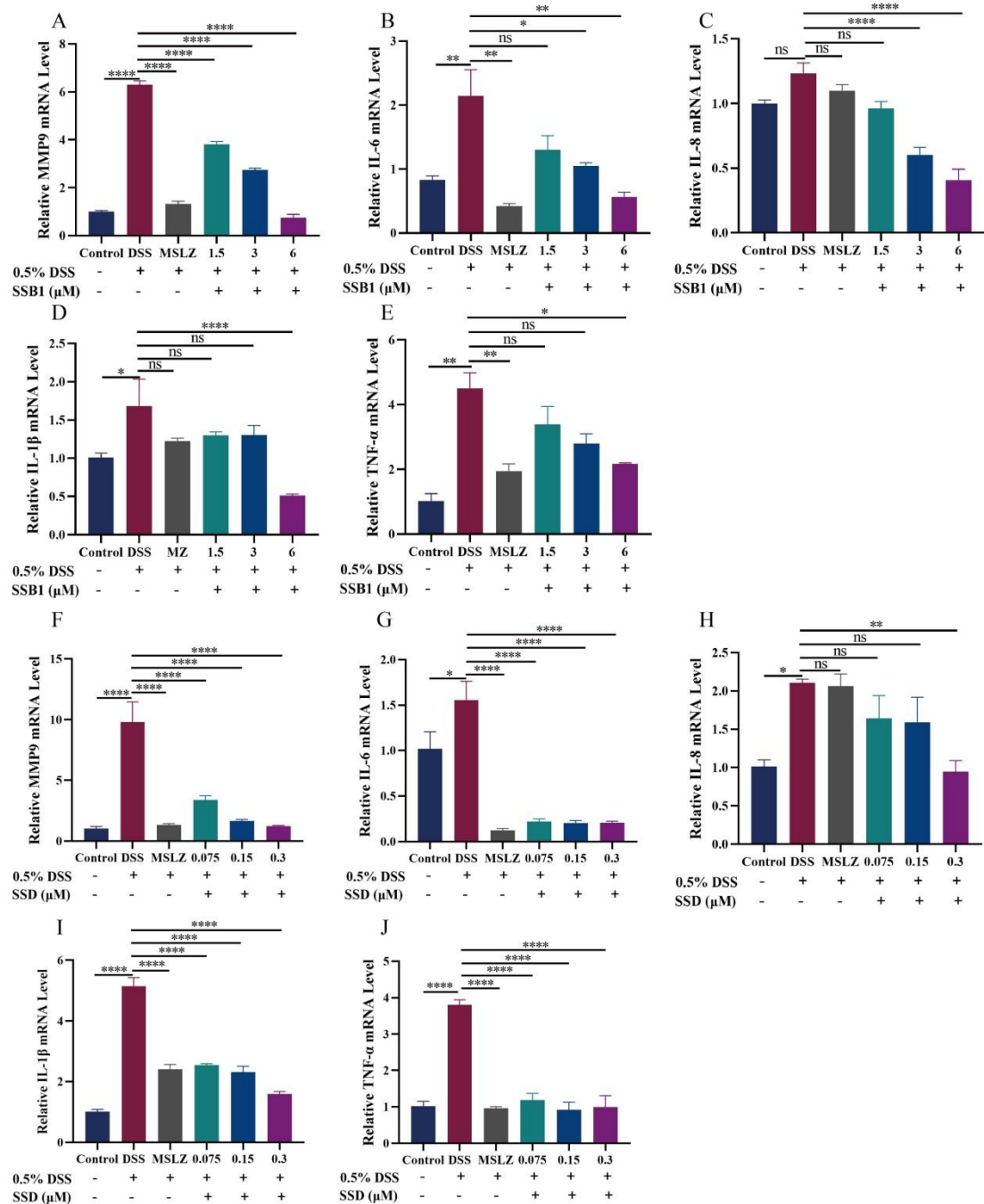


Figure. 4 SSB1 and SSD inhibits inflammatory cytokine production in DSS-induced colitis (A)The mRNA expression levels of (A) MMP9, (B) IL-6, (C) IL-8, (D) IL-1β, and (E) TNF-α in zebrafish tissue of colitis model with or without SSB1 treatment were estimated by RT-qPCR. (B)The mRNA expression levels of (F) MMP9, (G) IL-6, (H) IL-8, (I) IL-1β, and (J) TNF-α in zebrafish tissue of colitis model with or without SSD treatment were estimated by RT-qPCR. Statistical significance was assessed by one-way ANOVA. The results were presented as the means $\pm$ SEMs. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

3.5. SSB1 and SSD Regulated ROS level induced by DSS

We evaluated the effect of SSB1 and SSD on the levels of oxidative stress in DSS-stimulated zebrafish larvae. As shown in the fig.5, the fluorescence intensity in DSS group was significantly higher than that in the control group. Notably, the ROS generation in SSB1 and SSD-treated was decreased in a dose-dependent manner.

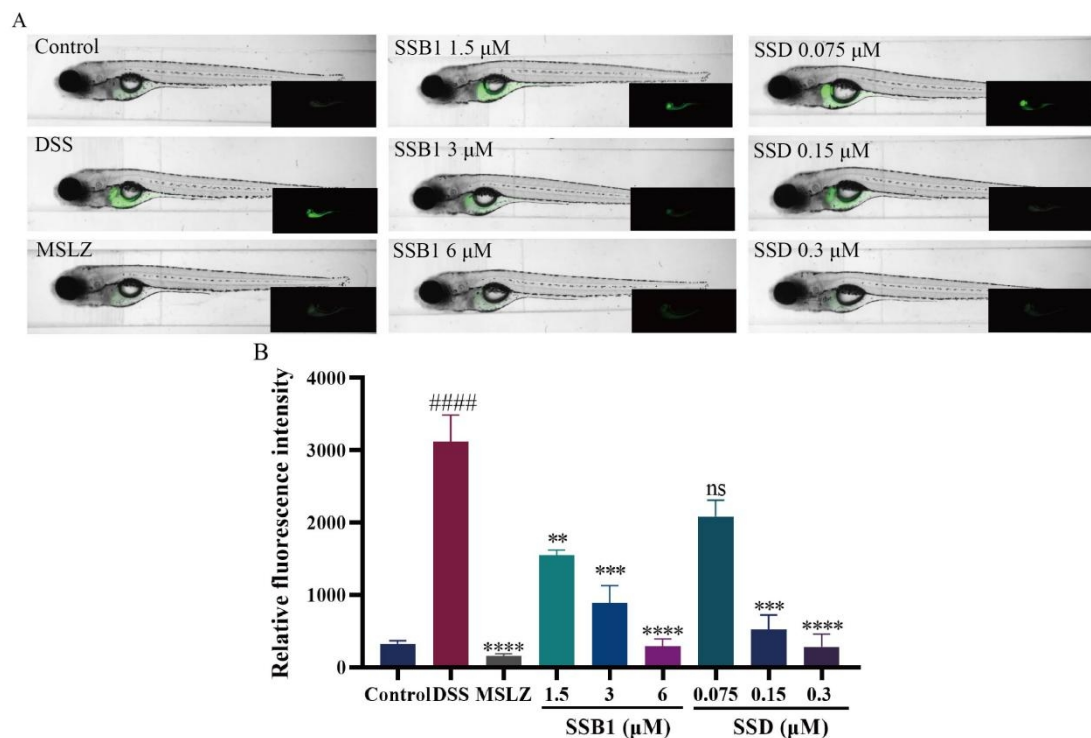


Figure. 5 ROS level are regulated by SSB1 and SSD. The 7 dpf zebrafish larvae were stained to evaluate the effects of DSS stimulation and SSs treatment on the level of oxidative stress (A) zebrafish microscope bright field and fluorescence image and merged image at 7 dpf. (B) Quantitative fluorescence intensity analysis of intestinal ROS content in dpf zebrafish larvae. Data were plotted by mean $\pm$ SEM. The control and DSS groups were compared using the unpaired t-test. One-

way ANOVA was used to test dose different groups to the DSS group. #### $p < 0.0001$ vs. the control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. DSS group.

3.6. SSB1 and SSD inhibited ferroptosis in DSS-induced zebrafish colitis

Previous studies have shown that suppressing ferroptosis can effectively ameliorate DSS-induced colitis [8,9,34,35]. Moreover, SSB2 treatment can exert anti-inflammatory effects by inhibiting ferroptosis [36]. Therefore, we further studied the effects of SSB1 and SSD on the ferroptosis-related markers in zebrafish tissues. As shown in Fig. 6, SSB1 and SSD treatment decreased the expression of PTGS2 and increased the expression of FTH and FTL in zebrafish tissues in a dose-dependent manner (Fig. 6A-C, G-I). Besides, MDA, GSH and iron load are three markers of ferroptosis [37]. We found that SSB1 and SSD administration increased the level of GSH and reduced the level of MDA and iron load in the zebrafish tissues after DSS challenge. (Fig. 6D-F, J-L). Taken together, SSB1 and SSD could inhibit ferroptosis by up-regulating NRF2/HO-1 signaling pathway (Fig.6).

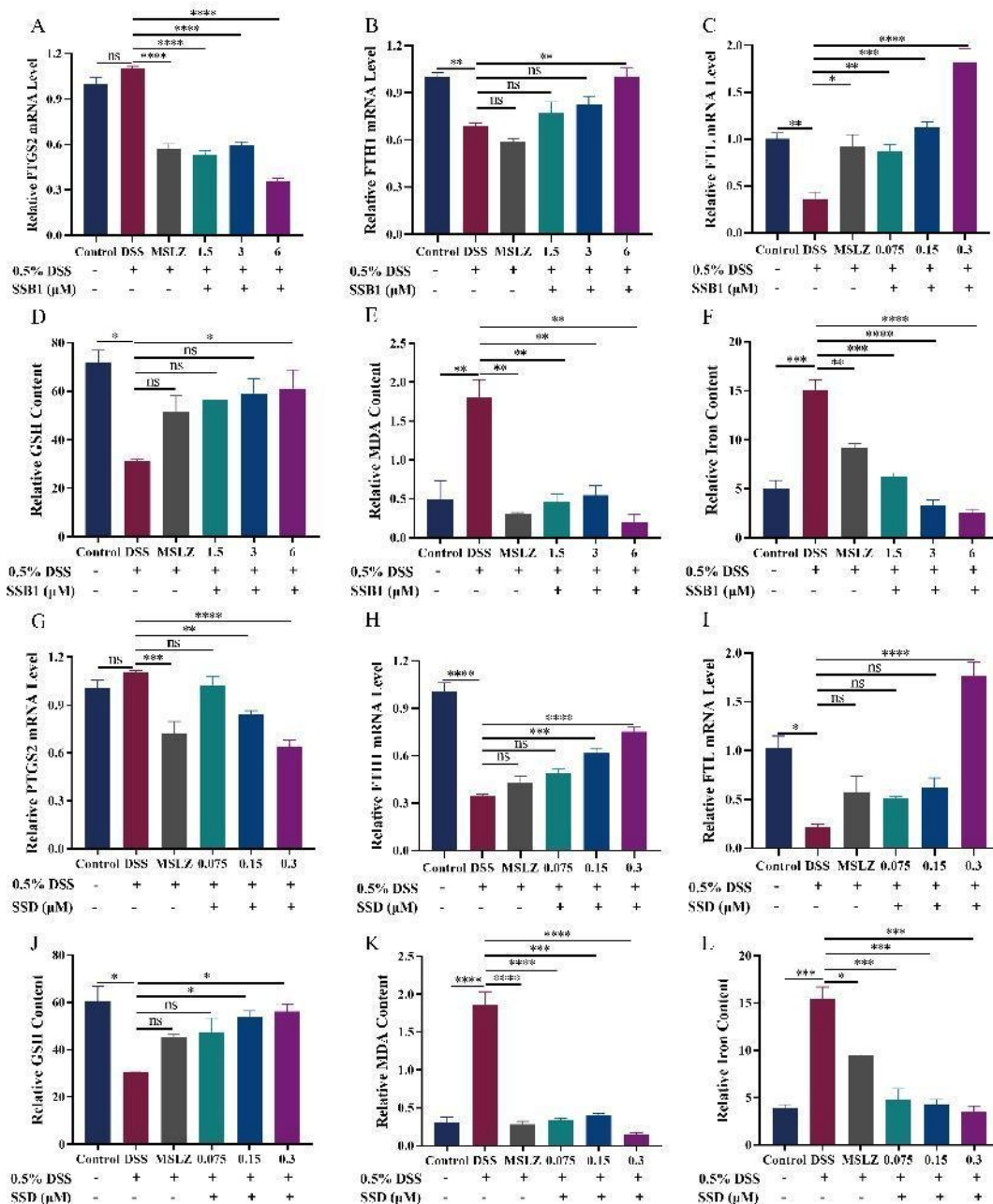


Figure. 6 SSB1 and SSD suppressed ferroptosis to attenuate DSS-induced UC. (A-C) The mRNA expression levels of PTGS2, FTH and FTL in DSS-treated zebrafish tissues with or without SSB1 treatment were detected by RT-qPCR. (D-F) The content of GSH, MDA and total iron contents in DSS-treated zebrafish tissues before and after SSB1 treatment. (G-I) The mRNA expression levels of PTGS2, FTH and FTL in DSS-treated zebrafish tissues with or without SSD treatment were detected by RT-qPCR. (N-P) The content of GSH, MDA and total iron contents in DSS-treated zebrafish tissues before and after SSD treatment. Statistical significance was assessed by one-way ANOVA. The results

were expressed as mean $\pm$ SEMs, N=30. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$.

3.7. SSB1 and SSD inhibited ferroptosis through the NRF2/HO-1 pathway

We further explored how SSB1 and SSD affected ferroptosis in DSS-induced colitis. SSD has been reported to inhibit the production of inflammatory mediators by activating the Nrf2/HO-1/ROS axis [38]. Suppressing ferroptosis could effectively ameliorate DSS-induced colitis, which is associated with the Nrf2/HO-1 signalling pathway [10]. In this study, we assumed that the SSB1 and SSD regulated ferroptosis in DSS-induced colitis through the Nrf2/HO-1 signaling pathway. As shown in the graph (Fig. 7), DSS induction led to a decrease in the expression of NRF2 and HO-1 in zebrafish tissues, while a dose-dependent increase in the expression of HO-1 and NRF2 in the SSB1 and SSD groups.

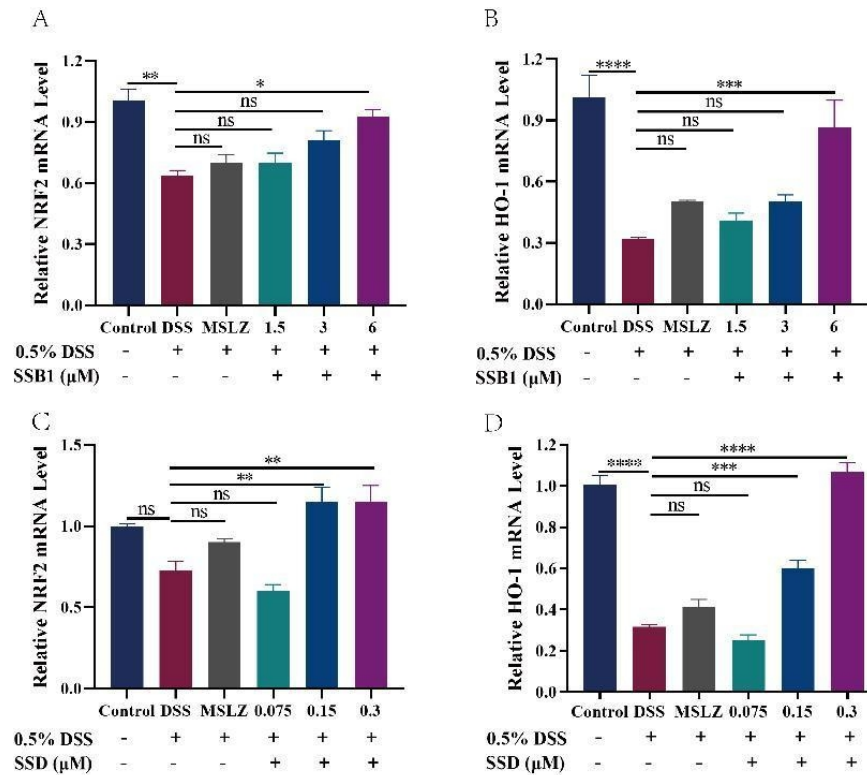


Figure. 7 SSB1 and SSD inhibited ferroptosis implicating with the Nrf2/HO-1 signaling pathway. The mRNA expression levels of NRF2, HO-1 in DSS-treated zebrafish tissues with or without SSB1 (A, B) and SSD (C, D) treatment were detected by RT-qPCR. Statistical significance was assessed by one-way ANOVA. The results were expressed as mean $\pm$ SEMs, N=30. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$.

3.8. Effects of SSB1 and SSD on morphology and neurodevelopment of zebrafish

In order to further investigate the potential toxicity of SSB1 and SSD during early development: morphological changes, mortality, body length, yolk sac area, hatchability, heart rate, and behavior of larvae treated with SSB1 and SSD were observed using stereomicroscope. Body length and yolk sac area are important indicators of embryos growth. Nutritional losses may result in shorter body length and accelerated yolk

sac absorption. Zebrafish larvae were exposed to different concentrations of SSB1, and the larval growth was measured at 96 hpf. As shown in Fig.8 B and C, SSB1 had no significant effects on the body length and yolk sac area of zebrafish compared to the control group. At the highest concentration of SSB1 (6 μ M), the hatching rate of embryos at 96 hpf was 98.3% (Fig.8 D). The hatching rate of the embryo is inversely proportional to the toxicity of the compound. As shown in Fig.8 D, the overall trend of hatchability of SSB1-treated embryos was little different from than that of control group. We further studied the toxicity to heartbeat and neurobehavioral development. It was found that high concentrations of SSB1 (6 μ M) showed a potential cardiac inhibition effect ($p < 0.001$), as shown in Fig.8 E. To evaluate the impact of SSB1 on the nerves of zebrafish, the effects of SSB1 treatment on touch-induced motion were assessed at 72 hpf. As shown in Fig.8 F. SSB1 exposure had no significant effect on the touch-induced escape response. And we measured the spontaneous movement frequency of embryos at 24 hpf. The results showed that SSB1 exposure had no significant effect on spontaneous movement, as shown in Fig.8 G. Therefore, we found that SSB1 had no obvious toxic side effects on the morphology and neurodevelopment of zebrafish.

As well, SSD had no apparent side effects on zebrafish morphology, cardiac and neurodevelopment. The results showed that SSD had low or

no acute toxicity to zebrafish embryos.

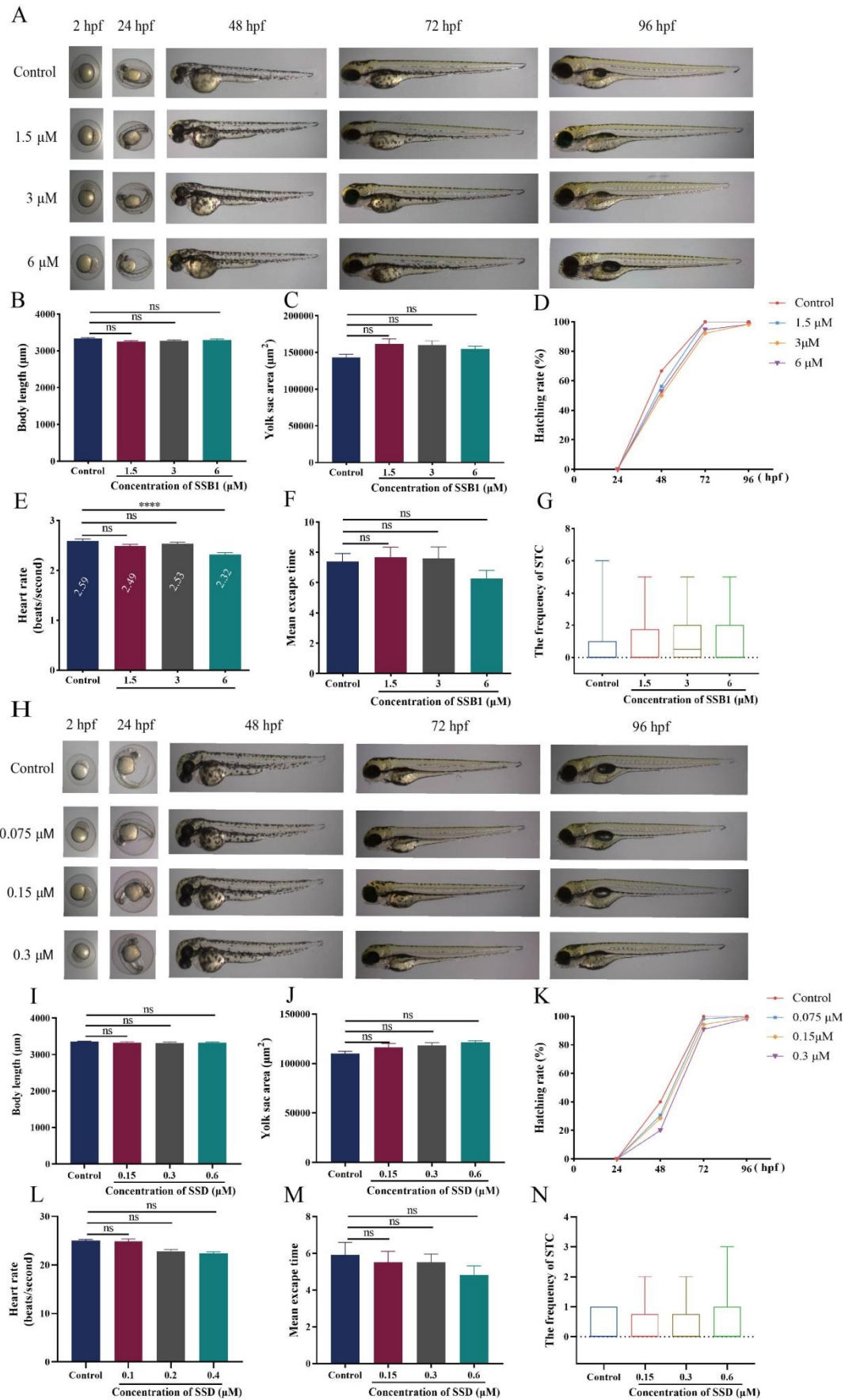


Figure. 8 Effects of SSB1 and SSD on zebrafish larvae development. (A) Representative photographs of morphological changes in embryos after 96 h of SSB1 intervention. Potential effects of SSB1 exposure on the (B) body length, (C) Yolk sac area, (D) hatching rate, (E) heart beat rate, (F) mean escape times and (G) spontaneous movement of zebrafish. (H) Representative photographs of morphological changes of in embryos after 96 h of SSD exposure. Potential effects of SSD treatments on the (I) body length, (J) Yolk sac area, (K) hatching rate, (L) heart beat rate, (M) mean escape times and (N) spontaneous movement of zebrafish. All data were plotted as means $\pm$ SEM. Statistical significance was assessed using one-way ANOVA. Values versus the control group: **** $p < 0.001$.

4. Discussion

IBD is a chronic lifelong inflammatory disease, and its intractability has become one of recognized medical challenges in the world. DSS induced intestinal inflammation in zebrafish has been well studied[31], characterized by the infiltration of neutrophils in intestinal tract [39]. Activated neutrophils release inflammatory cytokines, which is involved in the pathogenesis of colitis [40]. In addition, neutrophil derivatives mediate or exacerbate intestinal tissue damage [41]. Hence, therapeutic substances that reduce neutrophil recruitment may be helpful in reducing the inflammatory condition. So in this study, we constructed intestinal inflammation with DSS in the transgenic zebrafish Tg (mpx:EGFP^{il14}) expressing green fluorescent protein on neutrophils to study the anti-inflammatory effects of SSs. The results showed that SSA, SSB1, SSB2 and SSD were able to inhibit the recruitment of intestinal neutrophils. Remarkably, among them, SSB1 and SSD had the most

prominent effect, which demonstrated an excellent anti-inflammatory activity in vivo. But SSC and SSF had no significant effect on neutrophil recruitment. Previous evidence suggests that the resolution of inflammation was affected by the induction of neutrophil apoptosis and the reverse migration of neutrophils [42]. How SSs regulated the reduction of neutrophils to attenuate inflammation is unclear. Therefore, the effect of SSs on the retrograde chemotaxis and apoptosis of neutrophils deserves further investigation.

Currently, the common clinical therapies can't effectively control the progression of the disease and are accompanied by various side effects [13]. BC has been widely used to treat many diseases, such as colitis [43]. SSs is the main effective component of BC. A large number of studies have demonstrated that the SSs have anti-inflammatory effect. It has been reported that SSA can protect against DSS-induced colitis by inhibiting inflammatory response [27]. In this study, we determined the levels of pro-inflammatory cytokines in DSS-induced experimental colitis in zebrafish larvae. As expected, SSB1 and SSD were found to markedly reduced the secretion of inflammatory cytokines (MMP9, IL-6, IL-8, IL-1 β , and TNF- α), suggesting that SSB1 and SSD possess an anti-inflammatory effect in zebrafish.

Widespread ferroptosis has been reported in UC patients and DSS-induced experimental colitis [44]. Recent evidence suggests that

reducing ferroptosis significantly alleviates UC symptoms and facilitates intestinal restoration [34],[35]. In our current study, we explored the effect of SSB1 and SSD on ferroptosis in DSS-induced zebrafish colitis. Iron-catalysed lipid peroxidation is the main driver of ferroptosis, and massive accumulation of ROS is most prominent feature [45]. Previous studies have shown that iron chelators dramatically reduced ROS and improve clinical symptoms [46]. Herein, we observed that SSB1 and SSD reduced ROS accumulation, and promote intestinal repair. Moreover, our study showed that the expression of PTGS2 descended and the expression of FTH1 and FTL increased and the levels of GSH, MDA and iron load reversed to normal levels in SSB1 and SSD group. Nrf2 is considered to be a major transcription factor that regulates the expression of antioxidant proteins and blocks lipid peroxidation to alleviate oxidative stress [11]. Nrf2 has been widely proven to be participated in the pathological and physiological processes of UC [47]. Notably, the effect of NRF2 on ferroptosis has also been demonstrated [11]. Drugs that activate NRF2 can play a role in attenuating intestinal mucosal injury and intestinal inflammation by inhibiting ferroptosis [48]. The expression of HO-1 is significantly up-regulated in DSS-induced experimental colitis, thus exerting anti-inflammatory and anti-oxidative effects [49]. In this study, we found that the expression of NRF2 and HO-1 were downregulated in zebrafish tissues after DSS treatment, which

were upregulated after SSB1 or SSD administration. Together, we speculated that SSB1 and SSD were potential inhibitors of ferroptosis in DSS-induced colitis, which was associated with NRF2/HO-1 signaling activation. In addition, previous studies have found that SSD could exert anti-inflammatory activity through MAPK and NF- κ B signaling pathways, which are associated with ferroptosis [40,50], thus we do not rule out the possibility of SSD and SSB1 inhibiting ferroptosis through the above pathways.

Currently, conventional clinical use of IBD medications is associated with side effects such as poor maintenance effect, drug resistance, intolerance, systemic toxicity, dependence, loss of response and opportunistic infection. [13,51]. Chinese herbal medicine has been used in China for more than 2,000 years and is widely accepted due to its superior efficacy and low toxicity. BC has long been regarded as an effective and non-toxic herb. It was not until the emergence clinical reports of toxic damage caused by BC and its compound preparation in modern clinical application that the toxicity of BC was widely concerned and studied. Currently, the reported toxicological effects of SSD mainly include hepatotoxicity, neurotoxicity, hemolytic toxicity and cardiotoxicity [52]. We explored the developmental toxicity, neurotoxicity and cardiotoxicity of SSD and SSB1 on zebrafish embryos, and found that different concentrations of SSD and SSB1 had no

significant effects on the body length and yolk sac area of zebrafish. The effects of SSB1 and SSD on the hatchability of zebrafish were not much different from the control group. The SSB1 had an inhibitory effect on the heart rate at 6 μ M, while SSD had no significant effect on the heart rate. In terms of neurotoxicity, exposure to SSB1 and SSB1 had no significant effect on the touch-induced escape response and spontaneous movement. Therefore, we found that the effective concentrations of SSD and SSB1 had no significant toxic and side effects on the morphology and neurodevelopment of zebrafish.

5. Conclusions

In conclusion, our study demonstrated that SSB1 and SSD can alleviate DSS-induced experimental zebrafish colitis by inhibiting ROS accumulation and blocking ferroptosis, which is related to NRF2/HO-1 pathway activation.

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Statements & Declarations :

Author Contributions: G.-Q.Q.: supervision. H.-M.H. and K.-D.Z.: experiment performance, writing and original draft preparation, software, data sorting and collection. X.-Y.X.: experiment performance. B.-Y.L. and Q.-X.Y.: Assistance in experiment performance. H.-Z.Z. and Y.-P.J.: writing and editing. Z.Z.:review and editing □ S.-S.M. □ visualization□T.C.□project administration ; formal analysisAll authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The units declare no conflict of interest, especially Guangdong Longsee Biomedical Corporation has no conflict of interest with other units.

Figures

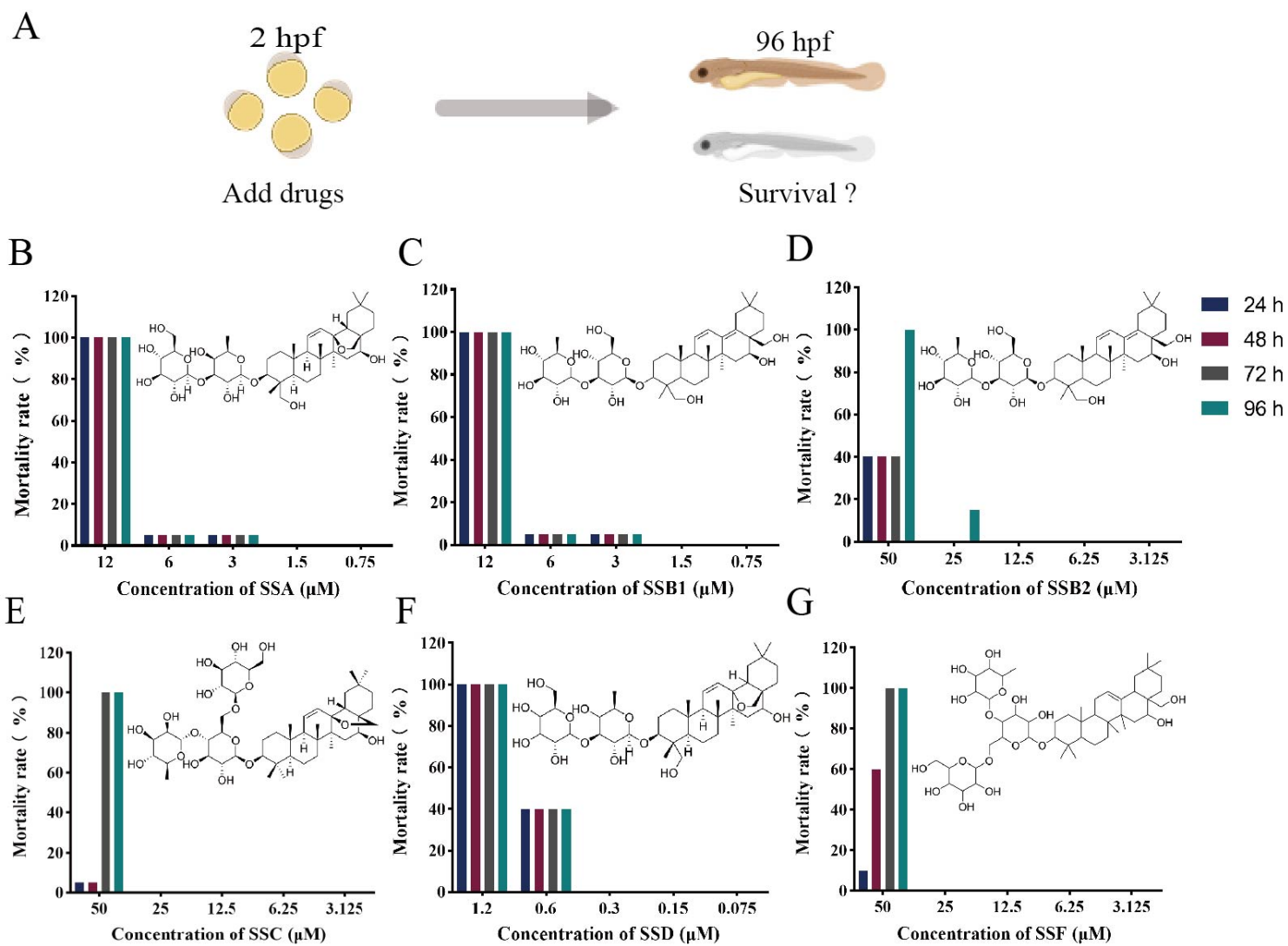


Figure 1



Figure 2



Figure 3

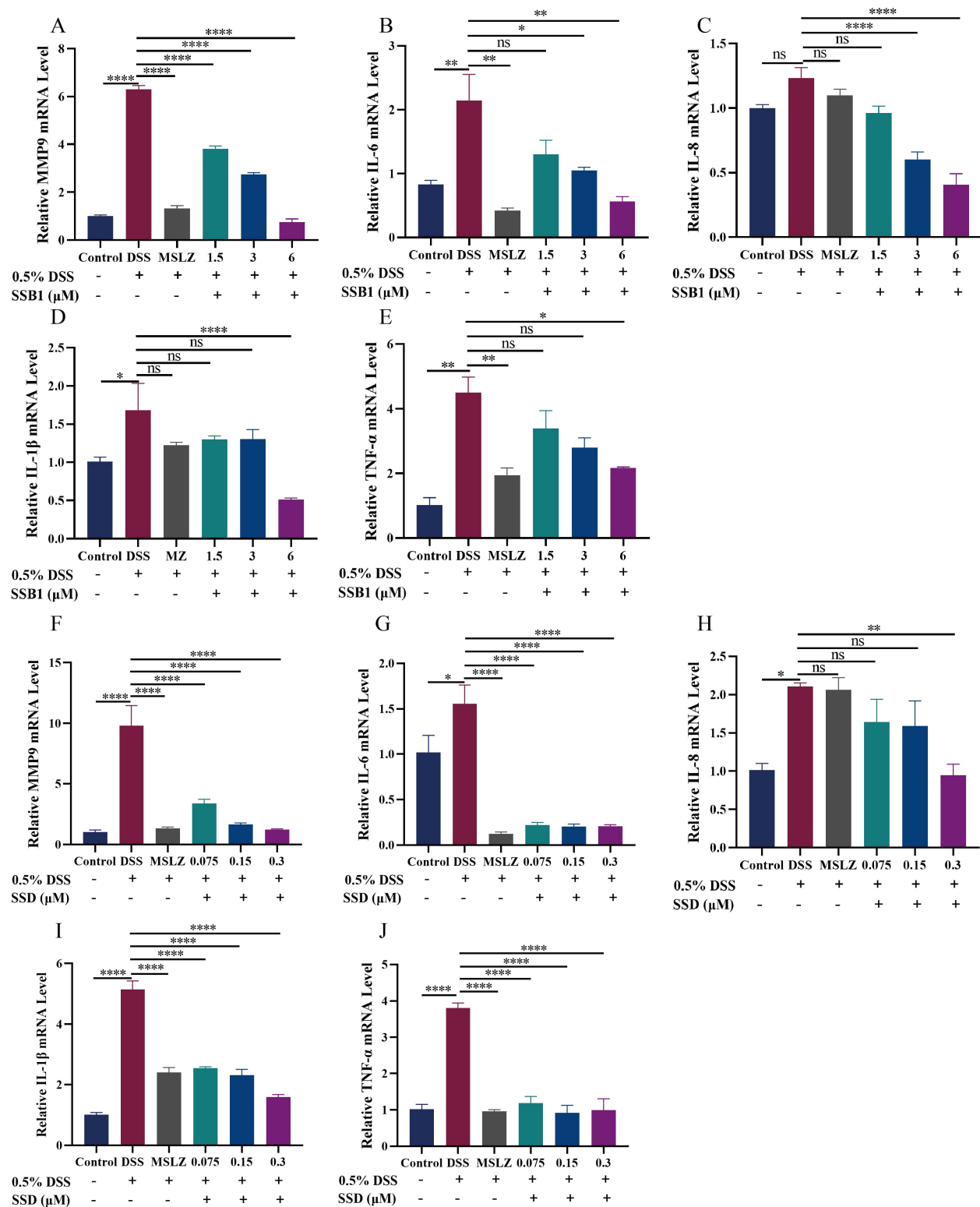


Figure 4

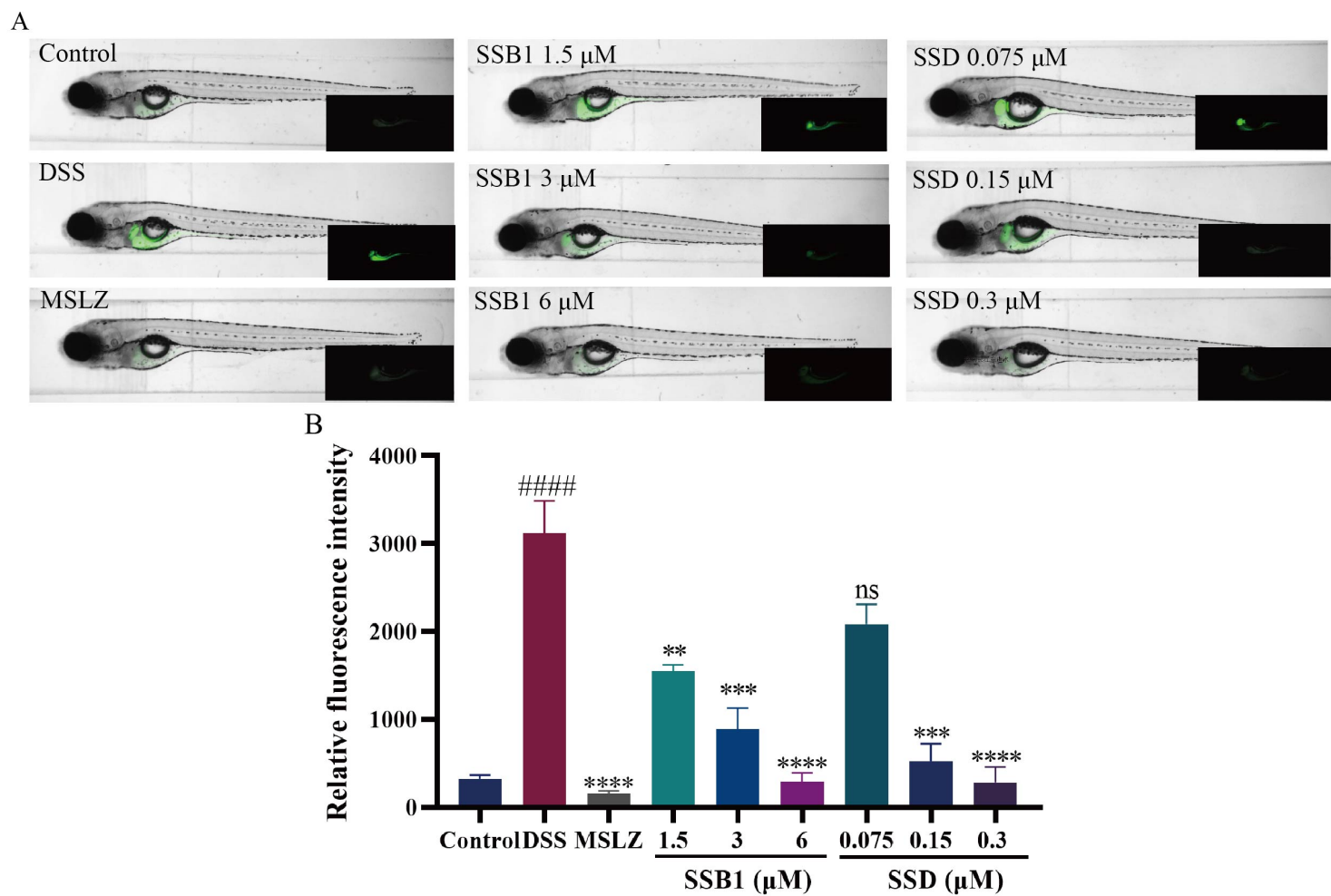


Figure 5



Figure 6



Figure 7

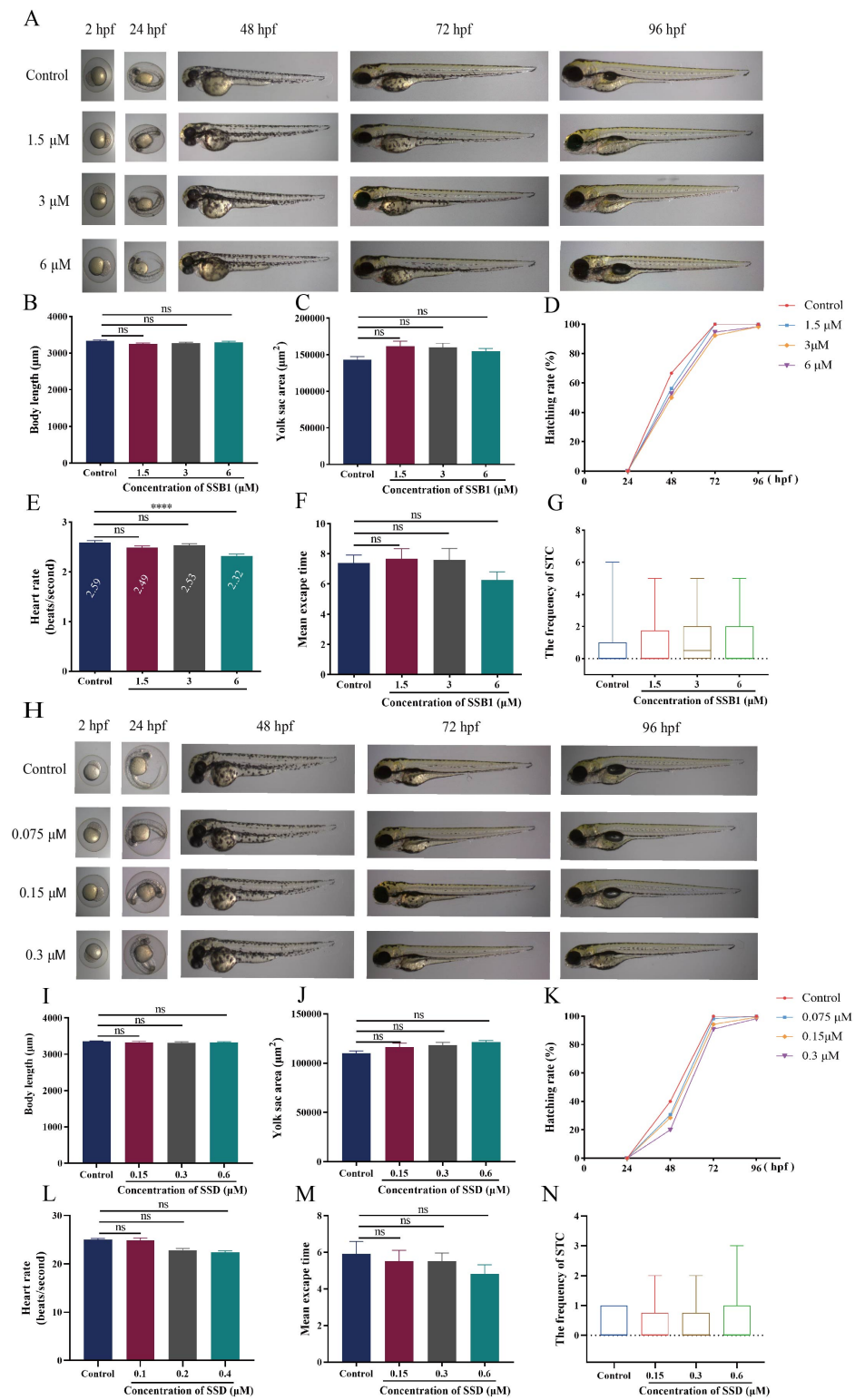


Figure 8

Supplementary Files

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- [QuantitativePCRprimersequences.docx](#)

- [FigureS1.tif](#)
- [FigureS1.tif](#)