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ARTICLE

Effect of Hirudin on farnesol X receptor pathway during acute intrahepatic cholestasis

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Abstract：**Objective:** The purpose of this study is to explore the effect of Hirudin on the farnesoid X receptor (FXR) pathway during acute intrahepatic cholestasis in vivo and in vitro. **Method:** In vivo, sixty male Sprague-Dawley rats were randomly divided into six groups: regular group, model group, ursodeoxycholic acid (UDCA) group (60 mg/kg), hirudin treatment group (84 u/kg), hirudin treatment group (63 u/kg) and hirudin treatment group (42 u/kg).The male Sprague-Dawley rats of UDCA group were intragastrically administered with a corresponding concentration of 0.005 mL/g body weight for seven days, once a day; and the hirudin treatment group was injected subcutaneously with different concentrations of Hirudin for seven days, once a day; Except for the normal group, other groups of rats were given 100 mg/kg ANIT by gavage on the 5th day. The model was administered by gavage once a day for three days. In vitro, (Z)-Guggulsterone was used to stimulate the L02 cells (0.05 μmol/ml), with or without different concentrations of Hirudin (2, 4 and 8 u/ml) for 24 h. The liver tissue was examined by HE microscope and the pathological state of the rat liver was observed; FXR, Small heterodimeric chaperone receptor (SHP), uridine diphosphate glucuronide transfer 2B4 (UGT2B4), bile salt output pump (BSEP)mRNA and protein expressions were tested by real-time fluorescent quantitative PCR and Western blot test. And immunohistochemistry (IHC) was used to analyze the expression of FXR. **Results:** Compared with the model group, the hirudin group can improve liver tissue damage, and promote FXR, SHP, BSEP and UGT2B4 proteins and mRNA expression in vivo and in vitro. **Conclusion:** Hirudin can alleviate intrahepatic cholestasis, reduce liver tissue damage. Hirudin can up-regulate the expression of FXR gene, promote the up-regulation of SHP, BSEP and UGT2B4 genes, and inhibit the cholestasis pathway to protect liver cells. The study may provide an effective drug for clinical treatment of intrahepatic cholestasis.

Keywords: hirudin; (Z)-Guggulsterone; α-isothiocyanate (ANIT); cholestasis; FXR

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Abbreviations: FXR， farnesoid X receptor; UDCA, ursodeoxycholic acid；SHP, Small hete rodimeric chaperone receptor；UGT2B4，uridine dip hosphate glucuronide transfer 2B4; BSEP, bile salt output pump; IHC，immunohistochemist；SAMe, s-adenosylmethionine; OC, obeticholic aci; ANI, alpha-naphthylisothiocyanate; GAPDH, glyceraldehyde-three-phosphate dehydrogenase; HRP, horseradish peroxidase; SPF, Specific pathogen-free; RIPA, radioimmunoprecipitation assay; BCA, bicinchoninic acid; PVDF, polyvinylidene fluoride.

Ethics approval and consent to participate: The present study was approved by The Institutional Animal Care and Use Committee of Hubei Provincial Hospital of Chinese Medicine.

Competing interests: The authors declare no competing interests.

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1. Introduction

Normal bile flow depends on the integrity of all structures, forms, and functions during the formation, secretion, and excretion of bile. When this integrity is compromised, a series of secondary reactions result in the formation and excretion of bile that is blocked, leading to intrahepatic cholestasis [1]. The current drugs used to treat cholestasis are mainly ursodeoxycholic acid (UDCA) and s-adenosylmethionine (SAMe), but these two drugs currently used in clinical practice have their shortcomings [2.3]. Therefore, we still need to continue to explore the treatment of intrahepatic cholestasis of effective drugs.

In the enterohepatic circulation, FXR regulates the gene network related to bile acid metabolism, controls many critical metabolic pathways, and maintains the homeostasis of bile acids. After FXR is activated, it affects the synthesis [4.5], detoxification [6] and transport [7] of bile acids to varying degrees. FXR agonists are classified by structure into steroids and non-steroid. The typical compounds in steroid agonists are the newly marketed new cholestatic liver injury treatment drugs obeticholic acid (OCA). In a mouse model of cholestasis, OCA promotes bile flow and protects liver cells from acute liver necrosis caused by low-density lipoprotein [8].

Traditional Chinese medicine for treating cholestasis is a characteristic and advantage of our country. It is more and more people's attention to find active substances against cholestasis from traditional Chinese medicine. Hirudin is the main component of the leech. Recent studies have found that Hirudin has anti-coagulation, anti-inflammatory, improved blood circulation and promotes angiogenesis [9-11]. Hirudin is by far the safest and most effective natural thrombin inhibitor found in the world.

However, whether Hirudin can relieve intrahepatic cholestasis needs further research. In this study, (Z)-Guggulsterone and ANIT were used to stimulate L02 hepatocytes and rats to build cell and rat models of intrahepatic cholestasis. Applying Hirudin to these cholestasis models assesses pathway changes in corresponding hepatocytes.

2. Experimental materials and methods

2.1 Reagents and chemicals

Hirudin (≥ 98% titration) was bought from the Chengdu AIKE Reagent Co, Ltd (Chengdu China). (Z)-Guggulsterone was bought from Sigma (St. Louis, USA). Alpha-naphthylisothiocyanate (ANIT) was bought from Sigma Reagent (STBC5577V); RNAiso Plus, PrimeScript RT reagent Kit and SYBR Premix Ex Taq kit were bought from the TaKaRa (Dalian, China). Rabbit anti-rat FXR、SHP、UGT2B4、BSEP and GAPDH were bought from the ProteinTech Group, Inc. (Wuhan, China), glyceraldehyde-three-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-labelled secondary antibody was bought from the Wuhan Boster Biotechnology Co., Ltd. (Wuhan, China).

2.2 Animal modelling and administration methods

Specific pathogen-free (SPF) male Sprague-Dawley rats weighing (200±20) g were bought from the Wuhan Municipal Center for Disease Control and Prevention, Animal License No. SCXK2015-0018. Forty male Sprague-Dawley rats were randomly divided into five groups: normal group, model group, UDCA group (60 mg/kg), hirudin treatment group (84 u/kg), hirudin treatment group (63 u/kg), hirudin treatment group (42 u/kg).The UDCA group was intragastrically administered with a corresponding concentration of 0.005 mL/g body weight for seven days, once a day. And the hirudin treatment group was injected subcutaneously with different concentrations of Hirudin for seven days, once a day; except for the regular group, rats of other groups were given 100 mg/kg ANIT tintragastrically on the 5th day of gavage. The model was administered by gavage once a day for three days. The rats were sacrificed 24 hours after the last administration, and take the liver tissue for correlation detection.

2.3 Cell culture and chemical treatment

The human L02 cell line was maintained in the DMEM with 10% FBS at 37°C . (Z)-Guggulsterone was used to establis cellular model. Intervention groups were divided into five groups: a model group, a UDCA group, and hirudin (8 u/mL, 4 u/mL, and 2 u/mL) groups. After the cells were cultured in a 6-well plate for 24 hours and the density reached 70%, then (Z)-Guggulsterone (0.05 μmol/mL) was used to stimulate the cells excluding the normal group. After 12 h, hirudin (8 u/mL, 4 u/mL, and 2 u/mL) was added into the wells except for the experiment group and control group. The supernatants and cells were harvested after 12 h.

2.4 Liver tissue HE staining and immunohistochemical detection

Take sections of rat liver tissues from each group for dewaxing, antigen repair, peroxidase binding, antigen blocking, and primary rabbit anti-rat FXR polyclonal antibody (concentration 1: 500). Secondary antibody goat anti-rabbit (concentration 1: 500). After DAB colour development, Mayer hematoxylin was used for counterstaining, dehydration, transparent, mounting. Application of Image-Pro Plus 6.0 digital medical

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image analysis system cumulative grey value of FXR brown-yellow positive expression particles.

2.5 Quantitative RT-PCR assays

According to the manufacturer's protocol, use RNA iso plus to isolate total RNA from LO2 cells or liver tissue and keep it at -80℃ before use. Perform denaturation, annealing, and amplification reactions on the RT-PCR System machine. The reaction conditions are as follows: pre-denaturation at 95 ℃ for 5 s; annealing at 60 ℃ for 20 s. The total reaction time is 1.5 h. The PCR reaction results were analyzed by Stepone software 5.0 software, and the β-actin was used as a reference for semi-quantitative analysis. Used $2^{-\triangle\triangle Ct}$ value for data analysis.The primer sequences were shown in Table 1.

2.6 Western blot analysis

The L02 cells or rat liver tissues were homogenized on ice by radioimmunoprecipitation assay (RI PA) lysis buffer (Beyotime Institute of Biotechnology) , and total protein was extracted. Bicinchoninic acid (BCA) protein analysis kit (Beyotime Institute of Biotechnology) was used to determine protein concentration. After 100g/L SDS-PAGE separation gel electrophoresis, polyvinylidene fluoride (PVDF) membrane transfer, skimmed milk powder blocking, the membranes were incubated with primary antibodies against FXR (1: 1000), SHP (1: 1000), UGT2B4 (1: 1000), BSEP (1: 1000), GAPDH (1: 1000) antibodies overnight at 4˚C. And incubated with secondary antibody (1: 5000) for 1 h, and then exposed with DAB solution. At last, used ImageJ software (version 3.0, LI-COR Biosciences) to analyze the gray value of the corresponding protein band.

2.7 Statistical analysis

Used SPSS software version 13.0 for all statistical analysis. The results were expressed as the means ± SD. The Student's *t*-test was used to show the comparison of the measured data between the two groups, and then a one-way analysis of variance (ANOVA) was performed by the Tukey test. $P<0.05$ was considered statistically significant. Use GraphPad Prism software (version 6) to draw graphs.

3. Results

3.1 Hirudin improves the pathological manifestations of the liver in rats with cholestasis

As shown in Figure 1, the liver lobule structure of rats in the regular group is clear. The cells line up nicely, and the nucleus of hepatocytes is round and bright. The liver cells of the model group are disordered, the core of liver cells shrinks, and there are a lot of inflammations. Sexual cell infiltration; UDCA group slightly relieved, hepatic lobule is brighter than structure, hepatocyte nucleus shrinks, visible foci necrosis, a little inflammatory cell infiltration; the hirudin 84u/kg group significantly diminished, visible liver tissue structure is complete The structure of hepatic lobule was clear, the number of hepatocyte necrosis decreased significantly; the hirudin 63u/kg group was slightly relieved, the hepatic lobular structure was intact, the number of hepatocyte necrosis decreased, and the hirudin 42u/kg group was compromised. Compared with the model group, the hepatocyte nuclei shrink, and a small numbert of inflammatory cells infiltrate.

Table 1:The primer sequences

	Forward primer （5'-3'）	Reverse primer （5'-3'）
β-actin(human)	TAGGAGCCAGG GCAGTAATCT	CGTTGACATCC GTAAAGACCTC
FXR(human)	TCCGCTGAACGA AGGAACAT	AAGTGACCTCC ACGACCAAGC
SHP(human)	AGATGTTCTTGA GGGTGGAAGC	GAAAGGCACTA TCCTCTTCAACC
BSEP(human)	CCACTCCAATCC CAGCAACT	ATGTTGACGGG ATTCGCTTC
UGT2B4(human)	GTATTGGGTCCT AAGGTGGGTG	CACTGCAAACC TGCTAAACCC
β-actin(rat)	TGCTGTCACCTT CACCGTTC	GTCCACCGCAA ATGCTTCTA
FXR(rat)	CTCCCTGCATGA CTTTGTTGTC	AAGAGATGGGA ATGTTGGCTG
SHP(rat)	TCTCCAATGATA GGGCGAAAG	GAAAGGGACCA TCCTCTTCAAC
BSEP(rat)	CATCCACTGCTC CCAACAAAC	CAACTGCTGGA CCGACAACC
UGT2B4(rat)	TTGTACAGCCGA GTATTGAGTCCT	GGTCGATGGTC AGTAACACGTC

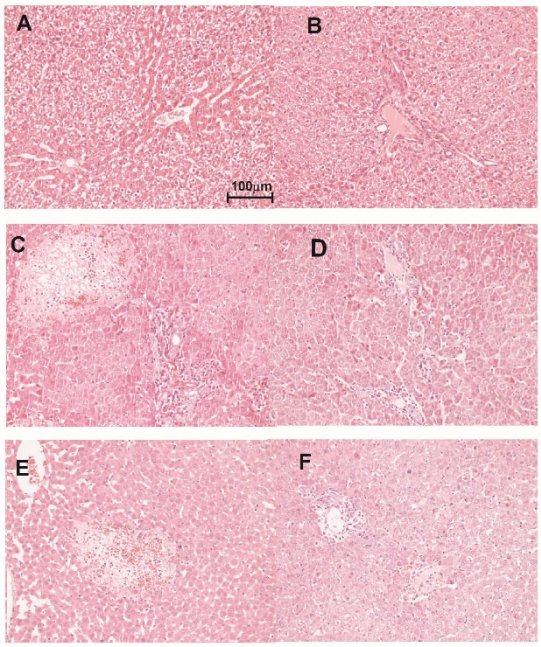

Fig.1 HE staining of liver tissue in rats with liver injury induced by ANIT (×100). A. Normal group B. Model group C. UDCA group D. Hrudin 84 u/kg E. Hirudin 63 u/kg F. Hrudin 42 u/kg

3.2 Hirudin increases the expression of FXR in the liver of cholestasis rats.

As shown in Figure 2, the favourable products of FXR protein staining are distributed in the cytoplasm. Compared with the regular group, the FXR protein expression in the model group was decreased (P<0.01). Compared with the model group, the hirudin 84u/kg treatment group, the hirudin 63u/kg treatment group, and the FXR protein expression was increased in the hirudin 42u/kg treatment group (P<0.01, or P<0.05), and FXR protein expression was raised in the UDCA group (P<0.01).

3.3 Relative expression levels of FXR, SHP, BSEP and UGT2B4 mRNA in liver tissue of each group.

As shown in Figure 3, compared with the normal group, the expression of FXR, SHP, BSEP and UGT2B4 mRNA in the model group decreased, which was statistically significant (P<0.05 or 0.01). Compared with the model group, the hirudin 84 u/kg treatment group, the hirudin 63u/kg treatment group and the UDCA group showed higher expression of FXR, SHP, BSEP and UGT2B4 mRNA statistically significant (P<0.05 or P<0.01). FXR, SHP, BSEP, UGT2B4 mRNA expression was increased in the hirudin 42u/kg treatment group, but no statistical significance.

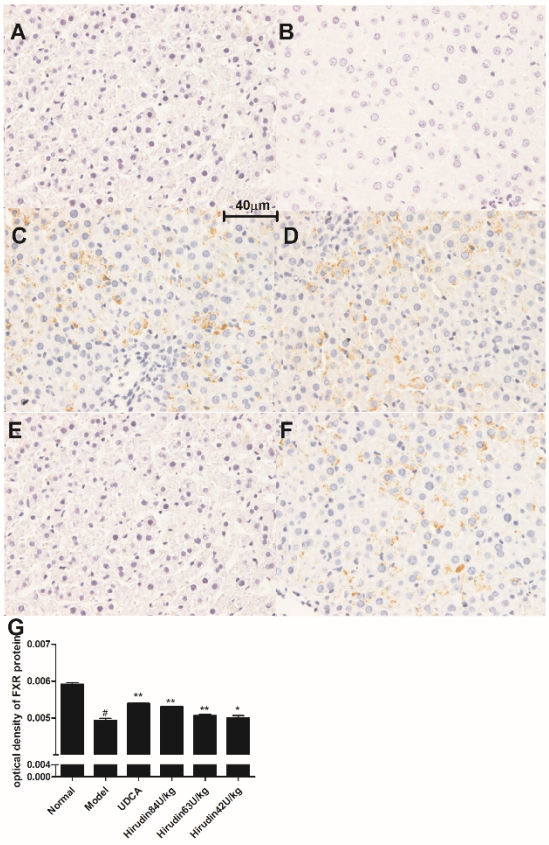

Fig.2 Hirudin enhances the expression of FXR mRNA in the liver of rats with cholestasis. n=3. #P<0.01 vs. normal; **P<0.01 vs model. *P<0.05 vs. model. FXR, farnesoid X receptor

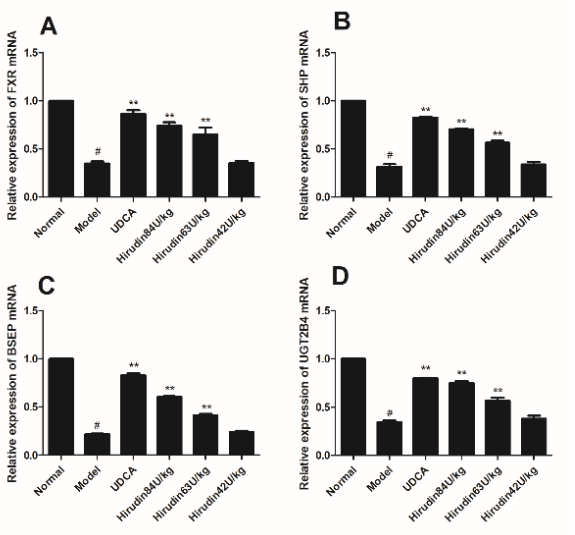

Fig.3 Hirudin enhances the expression of FXR(A), SHP(B), BSEP(C) and UGT2B4(D) mRNA in the liver of rats with cholestasis.n=3. #P<0.01 vs normal; **P<0.01 vs model. *P<0.05 vs. model. FXR, farnesoid X receptor; SHPS, small heterodimeric chaperone receptor; UGT2B4, uridine diphosphate glucuronide transfer 2B4; BSEP, bile salt output pump.

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3.4 Relative expession levels of FXR, SHP, BSEP and UGT2B4 protein in liver tissue of each group.

As shown in Figure 4, compared with the normal group, the expression of FXR, SHP, BSEP and UGT2B4 protein in the model group decreased, which was statistically significant (P<0.05 or 0.01). Compared with the model group, the expression of FXR, SHP, BSEP and UGT2B4 protein was increased in the hirudin 84u/kg treatment group, 63u/kg treatment group, 42u/kg treatment group and the UDCA group, which was statistically significant (P<0.05 or 0.01);

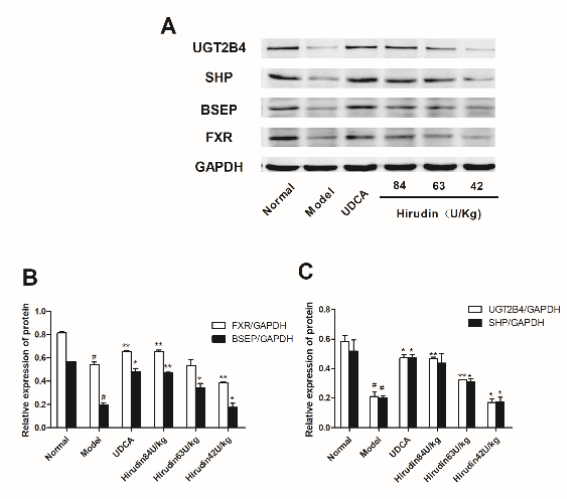

Fig.4 E Hirudin enhances the expression of FXR (A/B), SHP (A/B), BSEP (A/C) and UGT2B4 (A/C) protein. n=3. #P<0.01 vs. normal; **P<0.01 vs. model. *P<0.05 vs model. FXR, farnesoid X receptor; SHPS, small heterodimeric chaperone receptor; UGT2B4, uridine diphosphate glucuronide transfer 2B4; BSEP, bile salt output pump.

3.5 Relative expression levels of FXR, SHP, BSEP and UGT2B4 mRNA in L02 cells of each group.

As shown in Figure 5, compared with the normal group, the expression of FXR, SHP, BSEP and UGT2B4 mRNA in the model group decreased, which has statistical significance (P<0.05 or 0.01). Compared with the model group, the expression of FXR, SHP, BSEP and UGT2B4 mRNA was increased in the hirudin 8u/ml treatment group, 4u/ml treatment group and the UDCA group, which has statistical significance(P<0.05 or 0.01); FXR, BSEP, UGT3B4 mRNA expression was increased in the hirudin 2u/ml treatment group, which was not statistically significant, but the SHP mRNA expression was.

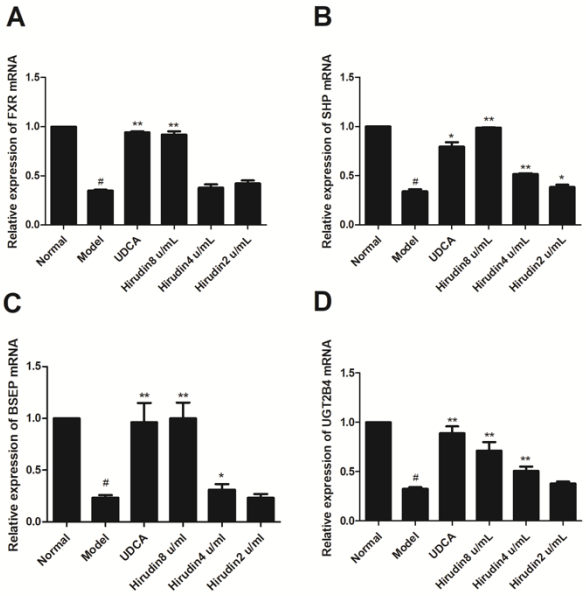

Fig.5 Hirudin enhances the expression of FXR(A), SHP(B), BSEP(C) and UGT2B4(D) mRNA in LO_2 cells stimulated by (Z)-Guggulserone. n=3. #P<0.01 vs. normal; **P<0.01 vs. model. *P<0.05 vs. model. FXR, farnesoid X receptor; SHPS, small heterodimeric chaperone receptor; UGT2B4, uridine diphosphate glucuronide transfer 2B4; BSEP, bile salt output pump.

3.6 Relative expression levels of FXR, SHP, BSEP and UGT2B4 protein in L02 cells of each group.

As shown in Figure 3, compared with the normal group, the hirudin 84 u/kg treatment group, the hirudin 63u/kg treatment group and the UDCA group showed lower expression of FXR, SHP, BSEP and UGT2B4 protein, which has statistical significance (P<0.05 or 0.01). Compared with the model group, the hirudin 8u/ml treatment group, 4u/ml treatment group and the UDCA group showed lower expression of FXR, UGT2B4, SHP and BSEP protein, which has statistical significance (P<0.05 or 0.01); the hirudin 2u/ml treatment group showed higher expression of FXR, SHP and UGT2B4 protein, which was statistically significant, but BSEP protein expression was not. However, the effect of the hirudin treatment group is not as good as the UDCA group.

4. Discussion

Cholestatic liver disease refers to obstacles to bile formation, secretion, and excretion caused by various causes inside and outside the liver, which prevents bile flow from flowing into the duodenum and flowing back into the blood. Clinical manifestations include jaundice, fatigue, itching, and deepening of urine colour. It is also accompanied by abnormal serum enzymes. Early asymptomatic symptoms, only manifested in elevated serum alkaline phosphatase and γ-glutamyl transpeptidase levels, hyperbilirubinemia can occur after the disease progresses, Severe cases

can lead to liver failure or even death [12]. In patients with chronic liver disease, the incidence of cholestasis is higher, followed by primary sclerosing cholangitis (67.00%), Autoimmune hepatitis (55.00%), and primary biliary cirrhosis (54.00%), Drug-related liver disease (53.00%), Chronic viral hepatitis (49.00%), cirrhosis (43.00%), alcoholic liver disease (35.00%) [13]. The purpose of drug treatment is to improve the clinical symptoms and liver damage caused by cholestasis. The main drugs are UDCA, SAMe and OCA [14]. Besides, FXR agonist NGM282 has entered PBC clinical phase II[15]. However, the efficacy of existing research drugs is unreliable, often accompanied by side effects, long synthesis cycle, and low success rate [16-18].

Traditional Chinese medicine to treat cholestasis is a characteristic and advantage of our country. It is more and more concerning to find active substances to improve cholestasis from concentional Chinese medicine. Hirudin is the main ingredient of parasite [19]. As a representative medicine for promoting blood circulation and removing blood stasis,the leech has the functions of Poxue, removing blood stasis, and passing menstruation.

At present, it has been found that nuclear receptors farnesoid X receptor (FXR) is the central link of bile component metabolism, and is currently considered as a critical target for the treatment of cholestasis [20-22]. It is considered to be a pivotal position in the treatment of cholestasis [23]. After FXR is stimulated, it mediates various pathways that inhibit cholestasis. Its function is network-like, and molecules downstream of FXR are regulated by multiple upstream signals. The literature shows [24-26]: FXR has unique effects on three different aspects of bile acid synthesis, detoxification and transport. In the bile acid synthesis pathway, the SHP-mediated inhibition pathway is a crucial molecule for the down-stream of FXR. In the bile acid detoxification pathway, UGT2B4 receives the influence from FXR, and transmits the detoxification signals of FXR, which is less affected by other signs. In the process of bile acid transport, FXR combined with the BSEP gene promoter can induce the expression of BSEP in the bile duct cell membrane and promote the secretion of bile acids. As can be seen, choosing FXR and downstream molecules closely related to FXR can observe the role and possible mechanism of interventions in the development of cholestasis. FXR agonists are a new class of drugs for the treatment of cholestasis. In addition to the newly marketed chemically synthesized drugs such as OCA [8,27], traditional Chinese herbal medicine extracts such as genocide [28], resveratrol [29]and alisol B 23－acetate, AB23A [30,31]can also improve cholestasis by activating FXR.

This study shows that, compared with the normal group, liver injury in the model group was severe, inflammatory cells infiltrated around the bile duct, some liver cells had oedema and necrosis, and FXR protein and mRNA expression decreased in vivo and vitro. It indicated that ANIT and (Z)-Guggulsterone had succeeded in modelling. UDCA was used as a positive control drug in this experiment. Compared with the model group, the liver tissue damage of rats in the hirudin treatment group was reduced, and the mRNA and protein expression of FXR was increased in each group, ownstream SHP, UGT2B4 and BSEP also performed the same. Compared with the hirudin treatment groups, the high-dose group had a significantly better effect on the mRNA and protein expression than those in the low-dose group. The hirudin treatment group can reduce the degree of liver damage caused by ANIT and reduce degeneration and necrosis of liver cells. The possible mechanism is to mediate the FXR-SHP, FXR-BSER, and FXR-UGT2B4 signalling pathways, thereby regulating the synthesis, metabolism, and detoxification process, thereby improving cholestasis. Still, the effect is not as good as UDCA.

In summary, hirudin can regulate the expression of genes related to bile acid synthesis, detoxification, and transport by promoting FXR expression, mediate inhibition of cholestasis pathways, and protect liver cells. In further experiments, we can consider studying the effect of combined use of UDCA and hirudin. This study provides a basis for hirudin to improve cholestasis.

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