Research Paper

Artesunate Inhibits Anti-Liver Fibrosis by Blocking the Epithelial-Mesenchymal Transition and Regulating the c-Jun N-Terminal Kinase Pathway

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Pan *et al.*: To Explore the Inhibition of Artesunate on Liver Fibrosis

To explore the inhibition of artesunate on liver fibrosis and to clear its mechanism of inhibiting anti-liver fibrosis by blocking epithelial-mesenchymal transition and regulating c-Jun N-terminal kinase pathway. Randomly take 18 male Sprague–Dawley rats. Set 6 untreated rats from them as control groups. Construct a rat model of liver fibrosis with another 12 rats and give intraperitoneal injection of a 50 % carbon tetrachloride olive oil solution for 1 ml/kg. After successful modeling, randomly divide them into model group and artesunate group, with 6 rats in each group. The artesunate group received 20 $mg.kg^{-1}.d^{-1}$ intragastric gavage and the rest 2 groups were given equal amounts of normal saline. Compare and analyze the function indexes of rat's livers (i.e. alanine aminotransferase and aspartate aminotransferase). Quantificational and in real time, detect the messenger ribonucleic acid expression of alpha-smooth muscle actin and collagen I in liver tissues by polymerase chain reaction and the expression of alpha-smooth muscle actin, collagen I, Slug, N-cadherin and c-Jun N-terminal kinase-related pathway protein by Western blot. The alanine aminotransferase and aspartate aminotransferase concentration of rat's serum in the artesunate group were obviously lower than that in the model group and the difference was of statistical significance ($p<0.05$). The messenger ribonucleic acid expression of alpha-smooth muscle actin, collagen I and protein content in the artesunate group were dramatically lower compared with the model group and the difference was of statistical significance ($p<0.05$). Masson staining tests showed that there was no obvious liver tissue fiber deposition in the control group and liver tissue was normal in morphology, while in the model group, a large amount of collagen fiber deposition was discovered in liver tissue. And the area of liver tissue collagen fibers obviously shrunk in the artesunate group compared with the model group. The content of Slug and N-cadherin in the hepatic tissue of artesunate group was much lower than that in the model group and the difference was of statistical significance ($p<0.05$). The protein content of p-c-Jun N-terminal kinase and p-c-Jun in hepatic tissue of the artesunate group was much lower than that in the model group and the difference was statistically significant ($p<0.05$). Artesunate can effectively inhibit the progress of liver fibrosis and the mechanism may have something to do with blocking the epithelial–mesenchymal transition and regulating the c-Jun N-terminal kinase pathway.

Key words: Artesunate, epithelial -mesenchymal transition, c-Jun N-terminal kinase pathway, liver fibrosis

Liver fibrosis is a chronic liver injury process and its causes may be relevant to long-term alcohol abusing, virus infection, obesity and familial inherited diseases[1]. Studies have shown that a variety of cells participate in the development of liver fibrosis, such as hepatocytes, cholangiocytes, bone marrow-derived cells and especially Hepatic Stellate Cells (HSC)[2]. During the pathological process, proinflammatory and profibrotic factors promote the activation and proliferation of these cells into myofibroblasts. These myofibroblasts produce excessive extracellular matrix that eventually leads to liver fibrosis and further liver cirrhosis, liver failure and even HSC[3]. The latest study considered liver fibrosis as a dynamic and reversible process[4] and found that retinoic acid and peroxisome proliferator-activated receptor gamma signaling pathway cooperate to reverse liver fibrosis[5], suggesting a reversible repair process for

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liver fibrosis. Targeting and alleviating liver fibrosis at an early stage is critical before developing into irreversible liver diseases like advanced cirrhosis and irreversible liver disease. Because there are currently no direct drugs for relieving or reversing liver fibrosis, new drug research and development has become a research hotspot. Artesunate is a semi-synthetic derivated product of artemisinin drugs and is one of the most commonly used for malaria. Artemisinin was extracted from Chinese medicine *Artemisia apiacea* by 2015 Nobel Prize winner Tu Youyou. Recent studies have found artesunate's attenuation on liver fibrosis. Artesunate could considerably depress the Hydroxyproline (HYP) content and the expression of Matrix Metalloproteinase (MMP)-2, MMP-9, alpha-Smooth Muscle Actin (α-SMA) and collagen I in the rat model of liver fibrosis. Presume that it inhibits the activation of HSC[6] with research on the inhibition of artesunate on liver fibrosis progress going further, this study intends to explore the effect of artesunate on Epithelial-Mesenchymal Transition (EMT) and c-Jun N-Terminal Kinase (JNK) signaling pathways, in order to offer a theoretical groundwork for the clinical treatment of liver fibrosis.

MATERIALS AND METHODS

Experimental reagents and materials:

Artesunate was purchased from Guilin Southern Pharmaceutical Co., Ltd.; Bicinchoninic Acid (BCA) kits were purchased from Shanghai Biyuntian Biotechnology Co., Ltd.; rabbit anti-mouse α-SMA antibody was purchased from Beijing Boosen Company; rabbit anti-mouse collagen I was purchased from Abcam Corporation, United States of America (USA); rabbit anti-mouse LC3B and beclin-1 antibody were purchased from protein tech.

Desktop high-speed centrifuges were purchased from American IEC micromax RF, Inc; cell incubators were purchased from Thermo Fisher and Fisher Technology Co., Ltd.; low-temperature refrigerators were purchased from China Haier Co., Ltd.; the Polymerase Chain Reaction (PCR) instruments were purchased from Eppendorf company, Germany; Paraffin slicers and tissue dehydrators were purchased from Leica, Germany; multi-function DP-71 microscopes and combined polarized light sheets were purchased from Olympus, Japan and the multifunctional micro plate readers were purchased from Tecan, Switzerland.

Experimental animals and grouping:

Select 18 male rats at age of 6 w and weighing 160~200 g and breed them at temperature of 18°~22°, humidity of 50 %~60 %, enabling them to drink water and feed freely. Randomly take 6 untreated rats as the control group and construct a rat model of liver fibrosis with another 12 rats after adaptive feeding for a week, intraperitoneal inject 50 % Carbon tetrachloride (CCl_4) olive oil solution for 1 ml/kg at the frequency of twice a week. After successful modeling, randomly divide them into model group and artesunate group, with 6 rats in each group. The artesunate group received 20 $mg.kg^{-1}.d^{-1}$ intragastric gavage and the rest 2 groups were given equal amounts of normal saline and killed after being fed 4 w.

Test methods:

Liver function index detection: Analyze rat serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) with a Mind ray BS-800M fully automated biochemical analyzer.

Quantificational and in real time, detect the liver tissues messenger Ribonucleic Acid (mRNA) expression of α-SMA and collagen I by PCR. Prepare cell suspension and adjust the density to 1×10^6 cells/ml. Add 1~2 ml of cell suspension to every well of six-well plates. After cells adhered to the wall evenly, abandon the supernatant, shower cells with Phosphate-Buffered Saline (PBS) and treat them with Dimethyl-Ketoglutarate (DMKG) at 1 mM and 8 mM concentrations that did not affect cell survival rate. Treat the normal control group with PBS solution. After 24 h, extract RNA to perform PCR reactions quantitatively and in real time.

Detection of the expressing of α-SMA, collagen I, Slug, N-cadherin and JNK-related pathway proteins in rat liver tissues with Western blot: Cell culture intervention methods are the same as that in total cell protein extraction. Rinse cells 2 to 3 times with PBS and add 0.5~1 ml of the protein lysate to each well and mix well. Detect protein concentration with BCA after centrifugation.

Kill the experimental rats after 4 w molding and take specimens of the liver right lobes and fix them in 4 % paraformaldehyde solution for 24 h. Test the effect of DMKG on hepatic stellate cell fibrosis by Masson staining. Routinely dewax paraffin sections in distilled water and stain them with Masson

composite staining for 5~10 min. After washing with 0.2 % acetic acid 1 % phosphotungstate acid and 0.2 % acetic acid solution, dehydrate them with gradient alcohol. Observe under a light microscope after transparentizing and sealing.

Statistical methods:

Statistical data were analyzed with Statistical Package for the Social Sciences (SPSS) 20.0 software. Measurement data in this study were consistent with normal distribution and expressed with ($\bar{x}\pm s$), compare data between multiple groups by single-factor variance analysis, conduct comparison among groups by Least Significance Difference (LSD)-t statistical analysis. The difference was of statistical significance ($p<0.05$).

RESULTS AND DISCUSSION

The serum ALT and AST content in the model and artesunate groups were noticeably more than that in the control group ($p<0.05$) and less than that in the model group ($p<0.05$). The difference was statistically significant ($p<0.05$) as shown in Table 1.

Compared with the control group, the mRNA expression and protein content of α-SMA and collagen I in the model group were noticeably increased, the difference was statistically significant ($p<0.05$), while that in the artesunate group decreased. The difference was statistically significant ($p<0.05$) as shown in Table 2.

Masson staining tests showed that there was no obvious liver tissue fiber deposition in the control group and liver tissue was normal in morphology, while in the model group, a large amount of collagen fiber deposition was discovered in liver tissue. And the area of liver tissue collagen fibers obviously got smaller in the artesunate group when comparing it with the model group.

Compared with the control group, Slug and N-cadherin protein contents exceed far beyond in the model group ($p<0.05$) and that in the artesunate group were obviously lower. The difference was statistically significant ($p<0.05$) as shown in Table 3.

Differences in JNK protein expressing progress in each group were not statistically significant ($p>0.05$). Compared with the control group, p-JNK and p-c-Jun protein contents were absolutely more than that in the model group ($p<0.05$) and that in the artesunate group were obviously lower. The difference was statistically significant ($p<0.05$) as shown in Table 4.

TABLE 1: ANALYSIS OF SERUM LIVER FUNCTION INDEX DIFFERENCES OF ALL GROUPS OF RATS ($\bar{x}\pm s$)

Grouping	n	ALT (U/L)	AST (U/L)
Control group	6	70.65±8.08	145.87±18.62
Model group	6	137.43±24.72	214.36±24.89
Artesunate group	6	103.87±23.76	176.85±25.47

Note: Compared with 1~16 mM DMKG, $p<0.05$

TABLE 2: ANALYSIS ON mRNA AND PROTEIN EXPRESSION OF α-SMA AND COLLAGEN I IN LIVER TISSUES FROM EACH GROUP ($\bar{x}\pm s$)

Grouping	n	α-SMA		Collagen I	
		mRNA level	Protein level	mRNA level	Protein level
Control group	6	1.03±0.04	1.01±0.02	1.04±0.02	1.03±0.03
Model group	6	1.56±0.18	1.78±0.23*	1.73±0.21*	1.68±0.22*
Artesunate group	6	0.76±0.08*	0.67±0.09*	0.64±0.07*	0.73±0.09*

Note: Compared with the control group and the model group, *$p<0.05$

TABLE 3: ANALYSIS ON SLUG AND N-CADHERIN PROTEIN EXPRESSION IN LIVER TISSUES FROM EACH GROUP ($\bar{x}\pm s$)

Grouping	n	Slug protein	N-cadherin protein
Control group	6	1.02±0.03	1.04±0.05
Model group	6	1.43±0.18*	1.38±0.20*
Artesunate group	6	0.68±0.11*	0.53±0.12*

Note: Compared with the control group and the model group, *$p<0.05$

TABLE 4: ANALYSIS OF JNK PATHWAY RELATED PROTEIN EXPRESSION IN EACH GROUP ($\bar{x}$±s)

Grouping	n	JNK protein	p-JNK protein	p-c-Jun protein
Control group	6	0.76±0.06	0.42±0.08	0.23±0.05
Model group	6	0.79±0.07*	0.75±0.09*	0.74±0.08
Artesunate group	6	0.78±0.06*	0.48±0.06 *	0.32±0.05

Note: Compared with the control group and the model group, *p<0.05

Liver fibrosis is a compensatory relection to chronic liver injuries and inflammation caused by many kinds of chronic injuries. The current accepted associated mechanism is the Damage-Associated Molecular Pattern (DAMP)-Reactive Oxygen Species (ROS) and inflammatory mediators are released from injured hepatocytes, triggering nonspecific immune reactions. The liver Nuclear Factor kappa-B (NF-κB) induces kinase to be activated and lipid peroxides, Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) and hedgehog ligand are secreted from leukocytes. Together these chemistry cause activation of HSC and activated HSC leads to increased proliferation, scarring formation, contraction, reduction of matrix degradation and fibrosis[7], resulting in massive accumulation of extracellular matrix. It can be seen that HSC works in the process of liver fibrosis and perhaps effectively blocking liver fibrosis can be achieved by inhibiting HSC activation. And HSC inhibition can be realized by reversing the trans-differentiation of HSCs into myofibroblasts, cutting down the fibro activity of HSC and inducing the deaths or apoptosis process of HSC.

It's reported that sorafenib can function as a tyrosine kinase inhibitor to induce death of auto phagocytes on HSC *via* the Protein Kinase B (Akt)/mammalian Target of Rapamycin (mTOR)/p70S6K and JNK signal pathway[8]. Some studies have found that Tetrandrine can inhibit TNF-α and HSC activation. The mechanism is that it inhibits Transforming Growth Factor beta (TGF-β)-Activated Kinase-1 (TAK1) and JNK phosphorylation and it can reduce expression of α-SMA and TNF-Receptor-1-Concerned Death Domains (TRADD)[9]. In the Platelet-Derived Growth Factor (PDGF)-induced rat model of liver fibrosis, white peony root extracts (including phenol) has been proved to depress HSC migrating and collagen protein producing. This outcome is thought to be linked with the inactivation of extracellular signal-regulated kinase, p38 and JNK[10]. So the JNK pathway functions elementally in HSC activation and even in the development of liver fibrosis. As a component of the Mitogen-Activated Protein Kinase (MAPK) pathway, JNK has three isotypes, i.e. JNK1, 2, and 3. JNK1 and JNK2 are expressed in most types of cells, including endothelial cells, while JNK3 mainly exists in neuronal tissue[11]. JNK signal pathways are mainly activated by cytokines or being exposed to environment[12]. These extracellular stimuli trigger the activation of MAP, followed by phosphorylation of Mitogen Kinase Kinase 4 (MKK4) and MKK7, which are isotypes of mitogen activated protein kinase. MKK is a specific protein kinase that phosphorylates JNK at Thr183 and Tyr185. JNK exclusively phosphorylates the transcription factor c-Jun at two serine residues Ser63 and Ser73 in the N-terminal trans-activation domain. JNK and c-Jun make transcription factors active including activator protein 1, activating transcription factor 2, Elk-1, p53 and c-Myc, thereby regulating genes in the downstream involved in apoptosis, proliferating and differentiating[12]. Additionally, the JNK cascade reaction is regulated by Notch, NF-κB and any other signal pathway[13].

Artesunate (Anti-Retroviral Therapy (ART)), a sort of small molecule of artemisinin isolated from Chinese medicine *Artemisia apiacea*, drastically depresses the development of liver fibrosis, by down regulating bovine serum albumin-induced rat MMP2 and MMP9[14]. Nevertheless, there are merely a small amount of published studies having investigation on the anti-fibrotic properties of ART[15]. In Myelodysplastic Syndrome (MDS) cells (skeletal muscle-1 cells), ART regulates cell apoptotic by depressing the expressing of c-Myc and cyclin D1, the downstream targets of the Wnt/β-catenin signal pathways[16]. Artemisinin has also been clarified to depress Focal Adhesion Kinase (FAK), Akt and Glycogen Synthase Kinase-3 β (GSK-3 β). Artesunate inhibits HSC proliferating and activating and promotes cell apoptosis along the FAK/Akt/β-catenin pathways Lv[17]. One study indicated that artesunate could also have an anti-fibrotic function by depressing ferroptosis in the activated HSC[18]. This study found that the serum ALT and AST contents were dramatically decreased after the artesunate intervention in rats with liver fibrosis, which further

clarified the effect of artesunate against liver fibrosis. But it's unclear whether a new mechanism is involved. This study pointed out that Slug and N-cadherin contents in artesunate group were noticeably less than that in the model group and p-JNK and p-c-Jun protein contents in artesunate group lag behind that in the model group. Previous researches have confirmed functions and mechanisms of various molecules in the process of EMT participating in liver fibrosis and confirmed that TGF-β 1 promotes EMT in liver cells and accelerating the process of liver fibrosis. EMT process is involved in common liver pathways of liver fibrosis such as hedgehog signaling pathway, TGF-β signaling pathway and extracellular signal-regulated kinase signaling pathway[19]. The study found that Dihydroartemisinin (DHA) in high ROS cell microenvironment induced the phosphorylation of JNK1/2, a downstream target of ROS, increased the autophagy of activated HSCs, reduced the secretion of Interleukin (IL-4) and IL-6 inflammatory factors in cell supernatants, and further inhibited inflammation-induced HSC activation[20].

Based on above, artesunate can effectively inhibit the progress of liver fibrosis and the mechanism may be related to blocking the EMT and regulating the JNK pathway.

Author's contributions:

Cunwei Pan and Liangqi Li have contributed equally to this work.

Conflict of interests:

The authors declared no conflict of interests.

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