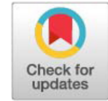
Pakistan J. Zool., pp 1-4, 2024. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20230804161622



Short Commucation

ω-3PUFA Inhibit Hepatic Fibrosis by Activating LKB1-AMPK Signal Pathway

Genfei Zhu, Xiaoqing Wu, Xianzhong Qian and Hong Hong*

Department of General Surgery, Zhejiang Hospital, Hangzhou, 310006, Zhejiang Province, China

ABSTRACT

Hepatic stellate cell (HSC) lines were cultured *in vitro* and treated with different concentrations of Ω-3PUFA. The effects of Ω-3PUFA on the survival rate of HSCs were detected by MTT, and the α-SMA, collagen I, TIMP-1 and MMP-13 proteins were detected by Western blot method. The rat model of hepatic fibrosis was established and divided into control group, model group and omega-3PUFA intervention model group. The effect of Ω-3PUFA on HSC fibrosis was detected by Masson staining, the levels of ALT and AST in serum were detected, and the relative proteins in liver tissue were detected by Western blot. Compared with the group A, 100 mm and 200 mm ω-3PUFA inhibited cell survival in a concentration-dependent manner, while 100 and 200mM ω-3PUFA decreased the expression of α-SMA, collagen Iand TIMP-1 protein in HSCs, while increased the expression level of MMP-13 protein in HSCs. The collagen fiber area of the liver tissue in the group C was reduced than that in the group B. The serum ALT and AST in the group B were raised, while these in the group C were reduced than those in the group A. The p-LKB1 protein in the group B was raised and the p-AMPK was reduced, while the p-LKB1 protein in group C was reduced and the level of p-AMPK was raised compared group A. Omega-3PUFA can significantly inhibit the activation of HSCs and reduce the degree of hepatic fibrosis by regulating LKB1-AMPK pathway.

Article Information
Received 04 August, 2023
Revised 05 October 2023
Accepted 18 October, 2023
Available online 18 March 2024 (early access)

Authors' Contribution
GZ and XW collected the samples. XQ and HH analysed the data. HH conducted the experiments and analysed the results. All authors discussed the results and wrote the manuscript.

Key words
ω- 3PUFA, LKB1-AMPK, Hepatic stellate cells, Hepatic fibrosis

Online First Article

Hepatic fibrosis refers to a chronic pathological change in which fibrous cells and matrix components proliferate, deposit and accumulate in the process of adverse injury, destruction and repair of normal liver cells, resulting in abnormal liver structure and function (Huang *et al*., 2017). The common causes include virus infection, alcoholism, fatty liver and so on. The clinical manifestations are hepatomegaly, ascites, jaundice and other symptoms. The course of disease is long, and it is easy to develop into liver cirrhosis and liver cancer, which brings serious harm to the life and health of patients. Liver fibrosis is one of the common pathological changes of chronic liver diseases, which can lead to serious consequences such as liver cirrhosis and liver cancer for a long time. At present, the prevention and treatment of liver fibrosis mainly include drug therapy, interventional therapy and surgical treatment, but the effect is not good, and there are many adverse reactions and side effects. As a result, liver fibrosis requires a safe and effective treatment.

Omega-3 polyunsaturated fatty acids (ω-3PUFA) exists widely in animals and plants. It can inhibit liver fibrosis in many ways (Koyama *et al*., 2017). Omega-3PUFA can inhibit liver fibrosis in many ways and has a certain therapeutic potential. ω-3PUFA can inhibit the proliferation, differentiation and collagen matrix synthesis of hepatic fibrotic cells, reduce the level of serum liver enzymes and improve the structure and function of liver (Hu *et al*., 2017). Although ω-3PUFA has become one of the research hotspots in the treatment of liver fibrosis, its mechanism is not completely clear. LKB1-AMPK signal pathway is one of the important mechanisms to inhibit liver fibrosis. LKB1 is a good AMPK kinase, which can directly activate AMPK and participate in the regulation of a variety of cellular metabolic processes. In the progression of hepatic fibrosis, the stimulation of inflammatory factors can promote the secretion of many kinds of cytokines and enhance the synthesis and accumulation of cross-linked collagen fibers, resulting in the proliferation and collagen deposition of fibroblasts. finally, the pathological changes

* Corresponding author: liangxian89856@163.com
0030-9923/2024/0001-0001 $ 9.00/0

Copyright 2024 by the authors. Licensee Zoological Society of Pakistan.
This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

of hepatic fibrosis were formed (Hu *et al*., 2017). This study is to explore the mechanism of ω-3PUFA on hepatic fibrosis, and we found that ω-3PUFA inhibiting hepatic fibrosis by activating LKB1-AMPK signal pathway.

Materials and methods

Rat hepatic stellate cells (HSCs) were purchased from San Francisco Hospital Hepatology Research Center, Japan from TCI company, fetal bovine serum from Gibco company, MTT kit from Sigma-Aldrich company, BCA kit (Shanghai, Biyuntian Biotechnology), rabbit anti-mouse α-SMA antibody (Beijing, Boosen), rabbit anti-mouse collagen I from (US, Abcam), and rabbit anti-mouse LC3B and Beclin-1 antibodies (Wuhan, Proteintech).

The desktop high-speed centrifuge was purchased from IEC microma x RF Company of the United States, the cell culture box was purchased from Seamer Fischer Technology Co., Ltd., the cryogenic refrigerator was purchased from Haier Company of China, the PCR instrument was purchased from Eppendorf Company of Germany, the paraffin slicer and tissue dehydration machine was purchased from Leica Company of Germany, the multi-function microscope DP-71 and combined polarizer were purchased from Olympus, Japan, and the multi-function enzyme labeling instrument was purchased from TECAN company of Switzerland.

Fifteen male rats, aged 6 weeks and weighing 160-200 g, were fed at a temperature of 18-22℃ and humidity of 50%-60%, and ate freely. After adaptive feeding for a week, 10 rats were randomly selected to establish a rat model of liver fibrosis, and intraperitoneal injection of 50% CCl_4 olive oil solution 1ml/kg was given twice a week. After successful modeling, the rats were randomly divided into model group and omega-3PUFA intervention model group with 5 rats in each group. The control group was only injected with the same amount of normal saline.

The effect of omega-3PUFA on the survival rate of HSCs was detected by 1MTT method: the cells were inoculated in a 96-well culture plate and made into cell suspension and counted according to the cell passage method. According to the results of cell count, the cells were diluted to 1×10 ~ 6/ ml, and then the cells were dispersed and mixed, and then a 96-well plate of 100 μl per well was added. PBS or acellular medium could be added around the plate. After the cells were uniformly adhered to the wall, the supernatant was washed with PBS, and the culture medium prepared by Ω-3PUFA containing 100 and 200mM concentration was added. Each concentration gradient was provided with at least 4 secondary pores. After 24 h of culture, PBS was washed, 20 μl of MTT solution was added, and incubated at 37℃ for 4 h. DMSO150 μ l was added to each well. The photometric value (OD) of each hole was detected by enzyme labeling instrument. The cell survival rate was [(group B (or C) - group A) OD value/ (normal control group-blank control group) OD value] × 100%.

The ALT and AST in serum of rats were detected according to the instructions of the kit. The effects of omega-3PUFA on α-SMA, collagen I, TIMP-1 and MMP-13 proteins in HSCs were detected by 3Westernblot. The total protein was extracted: the cells were washed with PBS for 2 or 3 times, the protein lysate 0.5~1ml was added to each well, and the protein concentration was detected by BCA after centrifugation.

The experimental rats were killed 9 weeks after modeling, and several specimens of the right lobe of the liver were fixed in 4% paraformaldehyde solution for 24 h. The effect of omega-3PUFA on HSC fibrosis was detected by Masson staining: paraffin sections were routinely dewaxed to distilled water, 5~10 min was stained with Masson, washed with 0.2% acetic acid solution, 1% phosphotungstic acid solution and 0.2% acetic acid solution, then the gradient alcohol was dehydrated and observed under light microscope after transparent and sealed.

Western blot was used to detect the effect of omega-3PUFA on the expression of LKB1, p-LKB1, AMPK and p-AMPK in liver tissue of rats in each group, and the method was the same as above.

The measurement data of this study are expressed by ($\bar{x}$±*s*). The comparison of the data of the two groups is tested by t test, and the comparison of the data of multiple groups by single factor analysis of variance is considered to have statistical differences. The data of this study are analyzed by SPSS21.0 software package. compared with the group A, $^aP < 0.05$; compared with 100 mM ω-3PUFA, $^bP < 0.05$

Results and discussion

Table I shows the ffect of ω-3PUFA on the survival rate, α-SMA, collagen I, TIMP-1 and MMP-13 in HSCs. The results of MTT assay showed that compared with the group A, 100mM and 200mM ω-3PUFA inhibited the cell survival rate and decreased the α-SMA, collagen I and TIMP-1 protein in HSCs, and increased the expression of MMP-13 protein in HSCs in a dose-dependent manner.

The results of Masson staining showed that there was a large number of collagen fibers in the model group, and the area of collagen fibers in the model group was reduced.

Table II shows the effect of ω-3PUFA on serological indexes of rats. Compared with the control group, the serum ALT and AST in the model group were raised, while the levels of ALT and AST in the group C were reduced than those in the control group. Moreover, the expression

Online First Article

of p-LKB1 protein in the group B was raised and the p-AMPK was decreased compared with control, while the p-LKB1 protein in group getting 200mm of ω-3PUFA was decreased and the p-AMPK was increased (Table II).

Table I. Effect of omega-3PUFA on the survival rate α-SMA, collagen I, TIMP-1 and MMP-13 in HSCs ($\bar{x}$±*s*).

Survival rate HSCs	Control (n=5)	ω-3PUFA (mM)	
		100 (n=5)	200 (n=5)
Survival rate (%)	101.85±5.73	66.82±1.76 [a]	49.67±0.694 [ab]
α-SMA	1.03±0.04	0.78±0.13 [a]	0.55±0.04 [ab]
Collagen I	1.02±0.04	0.82±0.09 [a]	0.65±0.12 [ab]
TIMP-1	1.07±0.05	0.76±0.04 [a]	0.58±0.06 [ab]
MMP-13	1.01±0.03	1.38±0.14 [a]	1.62±0.18 [ab]

Table II. Effect of ω-3PUFA on serological indexes of rats.

Indexes	Control (n=5)	ω-3PUFA (mM)	
		100 (n=5)	200 (n=5)
ALT (mmol/L)	32.85±5.28	286.95±36.52 [a]	112.74±13.68 [ab]
AST (mmol/L)	38.46±4.72	293.78±42.16 [a]	145.63±24.63 [ab]
LKB1	1.02±0.04	1.03±0.03	1.06±0.04
p-LKB1	1.02±0.04	1.48±0.16 [a]	1.23±0.10 [ab]
AMPK	1.03±0.03	1.02±0.03 [a]	1.04±0.04 [ab]
p-AMPK	1.03±0.04	0.67±0.07 [a]	0.86±0.08 [ab]

Discussion

Hepatic fibrosis refers to the development of chronic liver disease, in the liver, there is an abnormal distribution of excess extracellular matrix and repeated destruction of hepatocytes, which is the key step and important link in the development of liver cirrhosis (Zhao *et al*., 2016; Kuo *et al*., 2016; Tsuchida and Friedman, 2017). The further development of liver fiber will cause liver structural disorder, hepatocyte nodule regeneration leads to liver cirrhosis and reversible injury, but without active treatment, it is difficult to reverse and the prognosis is poor. Because the liver is one of the main metabolic and excretory organs of the body (Chen *et al*., 2017), Therefore, liver disease will have a huge negative impact on individual health and biological processes. When the liver is inflamed chronically, it gradually replaces its components with fibrous tissue, resulting in liver cancer (Wu *et al*., 2019), Therefore, liver disease will have a huge negative impact on individual health and biological processes. Liver fibrosis is the result of chronic inflammation of the liver caused by a variety of reasons, which makes the liver components gradually replaced by fibrous tissue, and eventually lead to liver cirrhosis, which can affect liver function and overall quality of life. Liver fibrosis is a gradually developing disease, so it is very important to diagnose and treat liver fibrosis as soon as possible. Liver fibrosis is a complex disease, and its pathogenesis involves a variety of cell types and molecular signal pathways. It has been found that a variety of cell types, including hepatocytes and stromal cells, are involved in hepatic fibrosis. in addition, specific signaling pathways, including apoptosis, cell proliferation, extracellular matrix deposition and changes in cell-matrix interaction, affect the development of hepatic fibrosis (Hu *et al*., 2017).

LKB1-AMPK is an important molecule to maintain the balance of energy metabolism (Homolya *et al*., 2014), it plays critical role in the process of cell metabolism. It is distributed in various organs and tissues of the body. CARLING proposed AMPK as an energy sensor for the first time and promoted human metabolism (Chen *et al*., 2017). Liver fibrosis, alcohol abuse, hepatitis virus infection, autoimmune disease, copper deposition disease and so on can occur under different conditions. When the liver is stimulated by these factors, it responds and releases a series of inflammatory mediators (Ceni *et al*., 2017), For example, inflammatory cells, oxidative stress, apoptosis and so on, resulting in hepatocyte injury, necrosis and component loss. These reactions can lead to the expression of endogenous molecules and transcription factors, such as TNF- α, IL-6 and so on. These molecules directly or indirectly promote hepatocyte injury and component loss, and accelerate the process of liver fibrosis (Cai *et al*., 2017).

During the occurrence of hepatic fibrosis, hepatocytes are stimulated, resulting in increased collagen deposition and fibrosis. As an energy sensor and regulator, LKB1-AMPK signal pathway plays an important role in metabolism and energy balance (Li *et al*., 2017). Compared with the group A, 100mm and 200mm ω-3PUFA inhibited cell survival in a concentration-dependent manner and decreased the expression of α-SMA, collagen I and TIMP-1 protein in HSCs, while increased the expression level of MMP-13 protein in HSCs. The liver tissue morphology was normal, while there were a large number of collagen fiber deposition in the group B. The collagen fiber area of the liver tissue in the group C was reduced than that in the group B. The serum ALT and AST in group C were reduced than those in group A. Compared with the group B, the p-LKB1 protein in group C was reduced and the p-AMPK was increased. This finding suggests that the protective effect of ω-3PUFA may be related to the activation of AMPK signal pathway. In addition, the

Online First Article

anti-fibrotic effect of ω-3PUFA may also be related to its anti-inflammatory effect. It has been found that the activation of LKB1-AMPK signal pathway can inhibit inflammation and oxidative stress, and reduce the damage of cells. Therefore, omega-3PUFA may reduce liver fibrosis caused by hepatitis virus infection by inhibiting inflammatory response. However, more studies are needed to explore the relationship between anti-fibrosis and inflammation in order to better understand its mechanism and provide more options and options for the treatment of liver fibrosis. ω-3PUFA has different therapeutic effects on different patients, and the therapeutic effect often varies from person to person, and liver fibrosis is a process of gradual development, which requires long-term treatment to achieve good results, and the mechanism of ω-3PUFA is not clear, so we need to continue to explore.

To sum up, omega-3PUFA can inhibit the progression of hepatic fibrosis by activating LKB1-AMPK signal pathway, which is helpful to further understand the protective effect of ω-3PUFA on hepatic fibrosis and its mechanism.

Acknowledgements

The authors are grateful to the support of Zhejiang Hospital, Hangzhou, 310006, Zhejiang Province, China.

Fundings

The research was supported by The Medical Science and Technology Project of Zhejiang Province (No. 2022RC095).

IRB approval

This research was carried out with the approval of Research Guidance Workshop Committee (Zhejiang Hospital).

Ethics approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Statement of conflict of interest

The authors have declared no conflict of interest.

References

Cai, C.X., Buddha, H., Castelino-Prabhu, S., Zhang, Z., Britton, R.S., Bacon, B.R. and Neuschwander-Tetri, B.A., 2017. *Dig. Dis. Sci.*, **62**: 968-978. https://doi.org/10.1007/s10620-017-4470-9

Ceni, E., Mello, T., Polvani, S., Vasseur-Cognet, M., Tarocchi, M., Tempesti, S., Cavalieri, D., Beltrame, L., Marroncini, G., Pinzani, M., Milani, S. and Galli, A., 2017. *J. Hepatol.*, **66**: 754-764. https://doi.org/10.1016/j.jhep.2016.11.003

Chen, C.H., Chen, M.F., Huang, S.J., Huang, C.Y., Wang, H.K., Hsieh, W.C., Huang, C.H., Liu, L.F. and Shiu, L.Y., 2017. *Am. J. Chin. Med.*, **45**: 351-368. https://doi.org/10.1142/S0192415X17500227

Chen, M., Liu, J., Yang, L. and Ling, W., 2017. *FEBS Open Biol.*, **7**: 811-820. https://doi.org/10.1002/2211-5463.12221

Homolya, L., Fu, D., Sengupta, P., Jarnik, M., Gillet, J.P., Vitale-Cross, L., Gutkind, J.S., Lippincott-Schwartz, J. and Arias, I.M., 2014. *PLoS One*, **9**: e91921(1-12). https://doi.org/10.1371/journal.pone.0091921

Hu, D., Hu, Y., Xu, W., Yu, H., Yang, N., Ni, S. and Fu, R., 2017. *Mol. Med. Rep.*, **16**: 1248-1254. https://doi.org/10.3892/mmr.2017.6702

Hu, X., Qiu, L., Liu, D. and Qian, L., 2017. *Med. Ultrason.*, **19**: 23-31. https://doi.org/10.11152/mu-942

Hu, Y.C., Liu, H., Liu, X.Y., Ma, L.N., Guan, Y.H., Luo, X. and Ding, X.C., 2017. *World J. Gastroenterol.*, **23**: 7425-7432. https://doi.org/10.3748/wjg.v23.i41.7425

Huang, Y., Deng, X. and Liang, J., 2017. *Exp. Cell Res.*, **352**: 420-426. https://doi.org/10.1016/j.yexcr.2017.02.038

Koyama, N., Hata, J., Sato, T., Tomiyama, Y. and Hino, K., 2017. *Hepatol. Res.*, **47**: 593-597. https://doi.org/10.1111/hepr.12776

Kuo, C.Y., Cheng, C.T., Hou, P., Lin, Y.P., Ma, H., Chung, Y., Chi, K., Chen, Y., Li, W., Kung, H.J. and Ann, D.K., 2016. *Oncotarget*, **7**: 34052-34069. https://doi.org/10.18632/oncotarget.8570

Li, B., Cong, M., Zhu, Y., Xiong, Y., Jin, W., Wan, Y., Zhou, Y., Ao, Y. and Wang, H., 2017. *Cell. Physiol. Biochem.*, **41**: 1481-1490. https://doi.org/10.1159/000470650

Tsuchida, T. and Friedman, S.L., 2017. *Nat. Rev. Gastroenterol. Hepatol.*, **14**: 397-411. https://doi.org/10.1038/nrgastro.2017.38

Wu, S., Liu, L., Yang, S., Kuang, G., Yin, X., Wang, Y., Xu, F., Xiong, L., Zhang, M., Wan, J. and Gong, X., 2019. *Immunopharmacol. Immunotoxicol.*, **41**: 438-445. https://doi.org/10.1080/08923973.2019.1613427

Zhao, J., Peng, L., Cui, R., Guo, X. and Yan, M., 2016. *Biochem. biophys. Res. Commun.*, **481**: 90-96. https://doi.org/10.1016/j.bbrc.2016.11.010

Online First Article