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Omega-3 polyunsaturated fatty acids prevent progression of liver fibrosis and promote liver regeneration after partial hepatectomy in cirrhotic rats

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Abstract. – OBJECTIVE: To assess the effect of omega-3 polyunsaturated fatty acids (n-3 PUFA) on liver regeneration of rats with liver cirrhosis after hepatectomy and antifibrosis.

MATERIALS AND METHODS: Omega-3 polyunsaturated fatty acids were intravenously injected in n-3 PUFA group 3 days before the operation to 1 day after partial hepatectomy. 70% hepatectomy was performed in rats, which were subsequently divided into 4 groups, namely normal and hepatectomy group (PH); liver cirrhosis and hepatectomy group (LC+PH); liver cirrhosis, n-3 PUFA (1 mL/kg), and hepatectomy group (LC+n-3 PUFA+PH); liver cirrhosis, n-3 PUFA (2 mL/kg) and hepatectomy group (LC+n-3PUFA*+PH). Body/liver weight ratios, serum parameters, histopathological examination, immunostaining, inflammatory cytokine and quantification of mRNA expression were also investigated.

RESULTS: Liver regeneration was significantly delayed compared with PH group 7 days after hepatectomy (PH) in LC+PH group. Besides, liver regeneration of LC+n-3 PUFA*+PH group increased significantly compared with LC+PH group 7 days after PH. In LC+PH group, liver cirrhotic was significantly higher compared with LC+n-3 PUFA+PH group 7 days after PH. In the meantime, liver cirrhosis of LC+n-3 PUFA*+PH group was significantly reduced compared with LC+n-3 PUFA+PH group 7 days after PH. Anti-inflammatory cytokine IL-10 was increased and pro-inflammatory cytokine IL-6 was decreased in LC+n-3 PUFA*+PH group compared with LC+PH group. N-3 PUFA also suppressed increments in mRNA expression for transforming growth factor-β and up-regulated the expression of matrix metalloproteinase-9 and matrix metalloproteinase-1 in the liver.

CONCLUSIONS: The mentioned results clearly show that n-3 PUFA reduces liver fibrosis and promotes liver regeneration, even under cirrhotic conditions. This could be a potentially useful treatment for liver cirrhosis.

Key Words:
Omega-3 polyunsaturated fatty acids, Liver cirrhosis, Liver regeneration, Inflammatory cytokines, Matrix metalloproteinase, Transforming growth factor-beta.

Introduction

Liver cirrhosis (LC) is considered a critical cause of morbidity and mortality worldwide, resulting in liver failure and portal hypertension and increasing the risk of carcinogenesis[1]. It is found in various chronic hepatic diseases after several types of injurious insult, e.g. viral infection, alcoholic and drugs[2]. In China, primary hepatocellular carcinoma (HCC) is usually associated with liver cirrhosis, and many studies[1,3] suggested that liver cirrhosis is the result of excessive accumulation of extracellular matrix (ECM) components, which consist of collagens and other components. Liver transplantation is now the only curative approach to severe liver cirrhosis[4]. The normal liver has a great capability to regenerate after partial hepatectomy; however, the regenerative abili-

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ty is so impaired that patients are at high risk of postoperative liver failure after hepatectomy in a cirrhotic liver[1]. Hepatic cirrhosis was historically considered an irreversible process due to the collapse of the hepatic parenchyma and its substitution with a collagen-rich tissue. Povero et al[5] have evidenced the reversibility of liver fibrosis in animal models. If a treatment that will restore these functions can be given to a cirrhotic liver in patients with advanced cirrhosis, postoperative liver failure may be avoidable.

Liver injuries can induce a cascade of inflammatory responses and as a result, initiate the process of liver fibrogenesis[6]. Hepatic stellate cells (HSC) are main ECM-producing cells which are activated by several key inflammation cytokines and liver fibrosis is associated with major alterations in both the quantity and composition of ECM[1,7]. N-3 PUFA is vital for clinical nutrition and nutrition support; it can also protect hepatocytes through inhibiting liver cells peroxidation *via* anti-oxidation mechanisms of action after PH[8]. Calder[9] indicates that n-3 PUFA and their specific lipid mediators can regulate the activity of inflammatory processes, which then suppress hepatocyte apoptosis and promote hepatocyte proliferation. Accordingly, we hypothesized that n-3 PUFA can not only promote liver regeneration but also improve liver fibrosis. The aim of this study was to investigate whether administration of n-3 PUFA improves liver fibrosis and liver regeneration under the condition of liver cirrhosis.

Materials and Methods

Animals

Sprague Dawley (SD) male weighing 180-220 g rats were purchased from Nanjing University (Nanjing, China). Rats were separated into four groups, namely normal and hepatectomy group (PH) (n=6), only received 70% partial hepatectomy; liver cirrhosis and hepatectomy group (LC+PH) (n=6), received CCl_4 and 70% partial hepatectomy; liver cirrhosis, n-3 PUFA (1 mL/kg) and hepatectomy group (LC+n-3 PUFA+PH) (n=6), received CCl_4 and 70% partial hepatectomy plus n-3 PUFA (1 mL/kg); liver cirrhosis, n-3 PUFA (2 mL/kg) and hepatectomy group (LC+ n-3 PUFA *+PH) (n=6), received CCl_4 and 70% partial hepatectomy plus n-3 PUFA (2 mL/kg). All the animal experiments were performed in a humane manner following the Guidelines of Nanjing University for the Care of Laboratory Animals and the Regulation for Animal Experiments of our University and Fundamental Guideline for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology. This investigation was approved by the Animal Ethics Committee of Nanjing University Animal Center.

Models for Liver Fibrosis and Surgical Procedure

To build a liver fibrosis model, each rat underwent an i.p. injection of CCl_4 (1 mL/kg body weight) twice a week for 8 weeks in a 1:3 ratio with corn oil. In those n-3 PUFA groups, rats were injected intravenously (*via* rat tail vein) with n-3 PUFA (Fish Oil, Fresenius Kabi Corp., Bad Homburg, Germany) at a dose of 1 mL/kg or 2 mL/kg body weight daily beginning from 3 days before the operation to 1 day after partial hepatectomy. 70% hepatectomy was performed in all groups. This procedure is to modify Higgins-Anderson operation[10] removing the left lateral, left median, and right median lobes with a single ligature. Hepatectomy was performed under ether anesthesia. The livers were removed and divided into two specimens: one aliquot was fixed in 10% buffered formalin for subsequent histological analysis, and the other aliquot was snap-frozen in liquid nitrogen and kept at -80°C until use.

Serum Parameters and Platelet Count

Blood samples were collected from the peripheral vessels in the quantity of 1-1.5 ml. Platelets were counted using MICROS abc LC-152 (Horiba Ltd., Kyoto, Japan). Blood samples were centrifuged for 10 min at 4°C at 3500 rpm. Supernatants were collected and stored at -80°C until tested using a serum multiple biochemical analyzer (Fuji Drichem; Fuji Film Inc., Tokyo, Japan) for measuring serum AST, ALT, and albumin levels.

Histology and Immunohistochemistry

Liver specimens fixed in 10% buffered formalin were employed for subsequent histologic and immunohistological analyses. Tissue sections were mounted on slides. Picrosirius red staining, Masson's trichrome staining, and alpha-smooth muscle actin (α-SMA) staining were preformed to study the extent of cirrhosis. For α-SMA staining, samples were incubated with anti-α-SMA (diluted 1:100; Dako, Glostrup, Denmark) antibody. The

liver fibrotic area was quantified using the winROOF visual system (Mitani Co., Tokyo, Japan).

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum were collected and stored at -80°C, and interleukin-6 (IL-6) and interleukin-10 (IL-10) were quantified using commercially available ELISA kits (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Serum IL-6 and IL-10 were measured in LC group and n-3 PUFA group.

Total RNA Extraction, cDNA Synthesis, and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Assay

Total RNA was extracted from liver tissue according to the Isogen method, reverse-transcribed using random primer, and SuperScript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA). QRT-PCR was performed in triplicate with the ABI PRISM 7300 Sequence Detection System (Applied Bio-Systems, Foster City, CA, USA). Relative quantification of gene expression was performed following the protocol of manufacturer, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as an internal standard. Primers of transforming growth factor-beta (TGF-β), matrix metalloproteinase-1 (TIMP-1), and matrix metalloproteinase-9 (MMP-9) are listed in Table I. Primers of TGF-β, MMP-9, and TIMP-1 were provided by Invitrogen Corp. (Carlsbad, CA, USA). The GAPDH primers were purchased from Taqman Gene Expression Assays.

Statistical Analysis

All data are expressed as the mean ± SD (standard deviation) of samples. The statistical analyses were performed with one-way analysis of variance (ANOVA), followed by the Post-Hoc Test (Least Significant Difference). In all cases, a p-value < 0.05 was considered significant.

Results

N-3 PUFA Promote Liver Regeneration in Liver Cirrhosis

The liver weight/bodyweight ratios of LC+ n-3 PUFA+PH and LC+ n-3 PUFA*+PH groups were increased compared with LC+PH group. LC+ n-PUFA*+PH groups were significantly increased compared with LC+PH groups 7 days after PH (Figure 1A). The liver weight: LC+ n-PUFA+PH and LC+ n-PUFA*+PH groups were increased compared with LC+PH group. LC+ n-PUFA*+PH groups were significantly increased compared with LC+PH groups 5 days after PH (Figure 1B).

N-3 PUFA Protect Liver Function in Liver Cirrhosis

Serum ALT levels were lower in LC+ n-PUFA*+PH groups with significant differences at 1 day and 3 days after PH in comparison with the LC+PH group (Figure 2A). Serum AST levels were lower in LC+ n-PUFA*+PH group with significant decreased at 1 day after PH in compari-

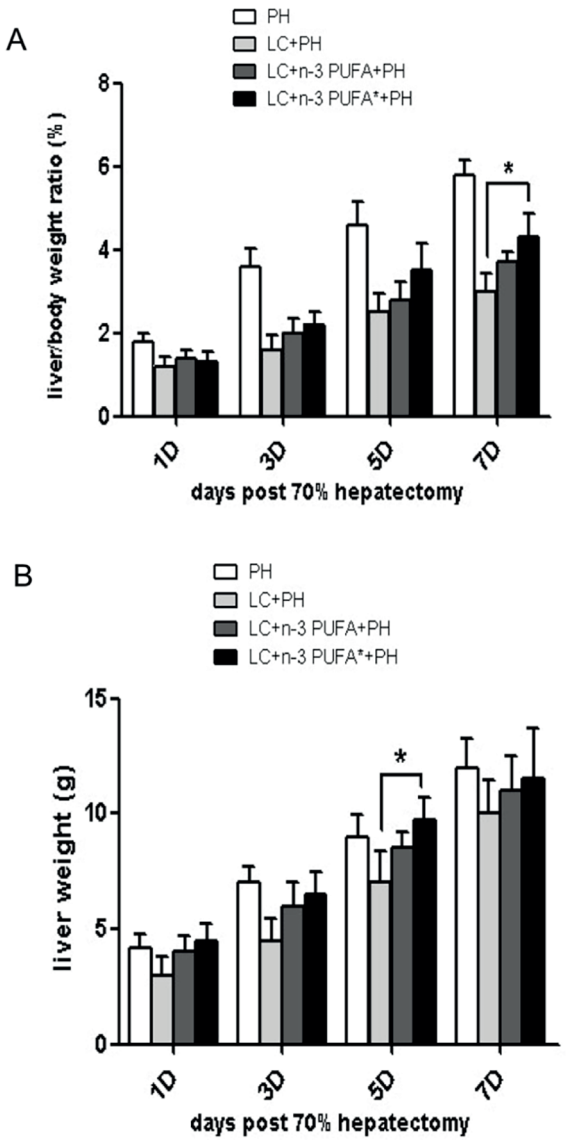

Figure 1. Effect of n-3 PUFA on liver regeneration after PH on liver regeneration of **(A)** liver/body weight ratio, **(B)** liver weight 1 day, 3 days, 5 days, and 7 days after PH. Data shown indicate the mean±SD, n=6 in each group. *p<0.05, *vs.* LC+PH group after PH.

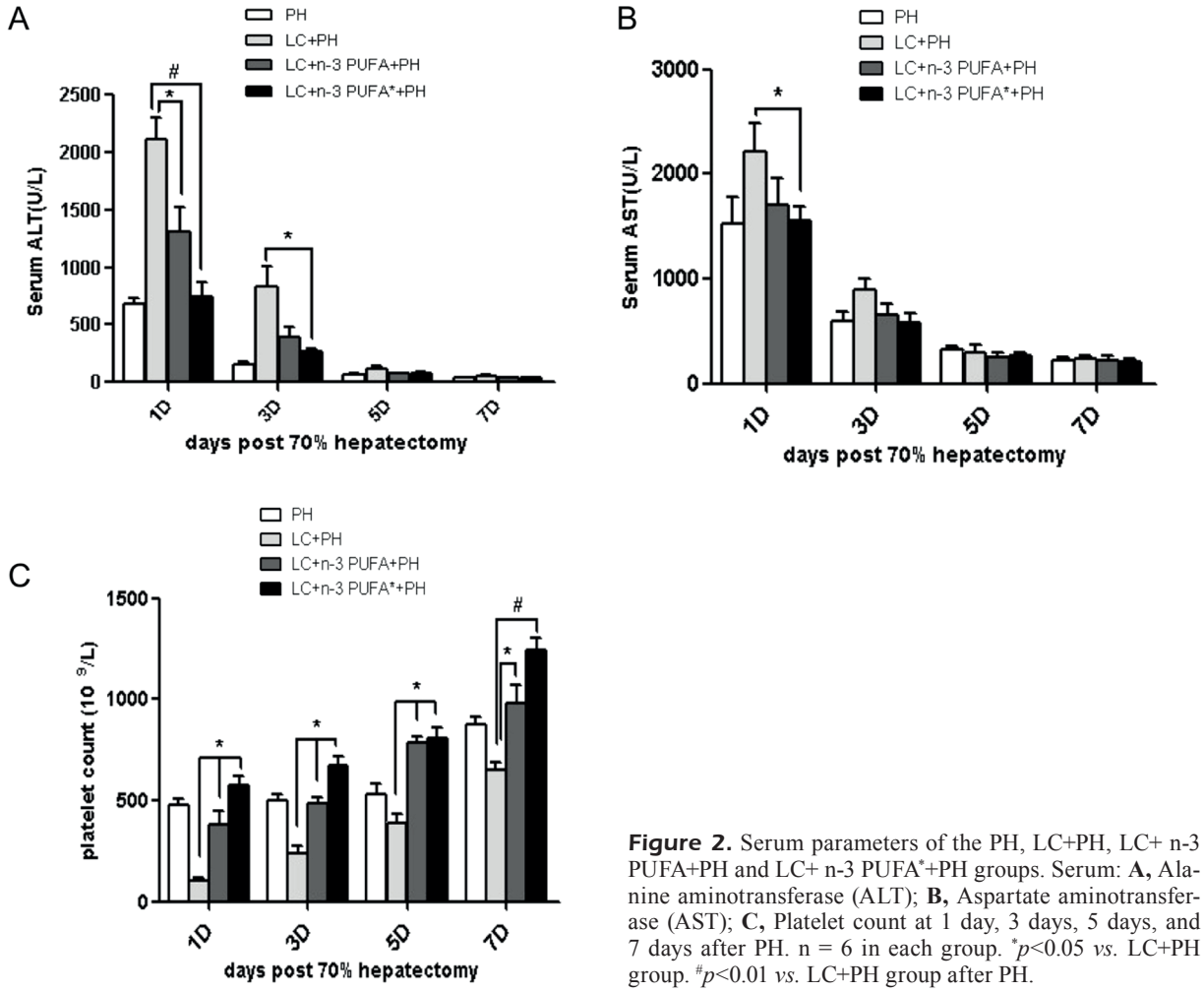

Figure 2. Serum parameters of the PH, LC+PH, LC+ n-3 PUFA+PH and LC+ n-3 PUFA*+PH groups. Serum: **A,** Alanine aminotransferase (ALT); **B,** Aspartate aminotransferase (AST); **C,** Platelet count at 1 day, 3 days, 5 days, and 7 days after PH. n = 6 in each group. $^{*}p<0.05$ *vs.* LC+PH group. $^{\#}p<0.01$ *vs.* LC+PH group after PH.

Table I. Primer sequences for TGF-β, MMP-9 ,TIMP-1, and GAPDH.

	Forward primer	Reverse primer
TGF-β	CTTCAGCTCCACAGAGAAGAACTGC	CACGATCATGTTGGACAACTGCTCC
MMP-9	GAAGACTTGCCGCGAGACGTGATCGATG	GCACCAGCGATAACCATCCGAGCGAC
TIMP-1	AATGCCACAGGTTTCCGGTTC	ACACCCCACAGCCAGCACTAT
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

son with the LC+PH group (Figure 2B). Platelet counts of the LC+ n-PUFA+PH and LC+ n-PUFA*+PH groups were significantly higher than the LC+PH group 1 day, 3 days, 5 days, and 7 days (Figure 2C). No significant differences were identified among all groups in the levels of albumin ($p>0.05$) (data not shown).

N-3 PUFA Reduce Cirrhosis Changes in the Liver

Progression of hepatic cirrhosis was monitored by Sirius red staining and Masson's trichrome staining, and activation of the HSC was followed by immunostaining for α-SMA. Sirius red staining in liver sections of all groups was assessed. Positive staining (red)

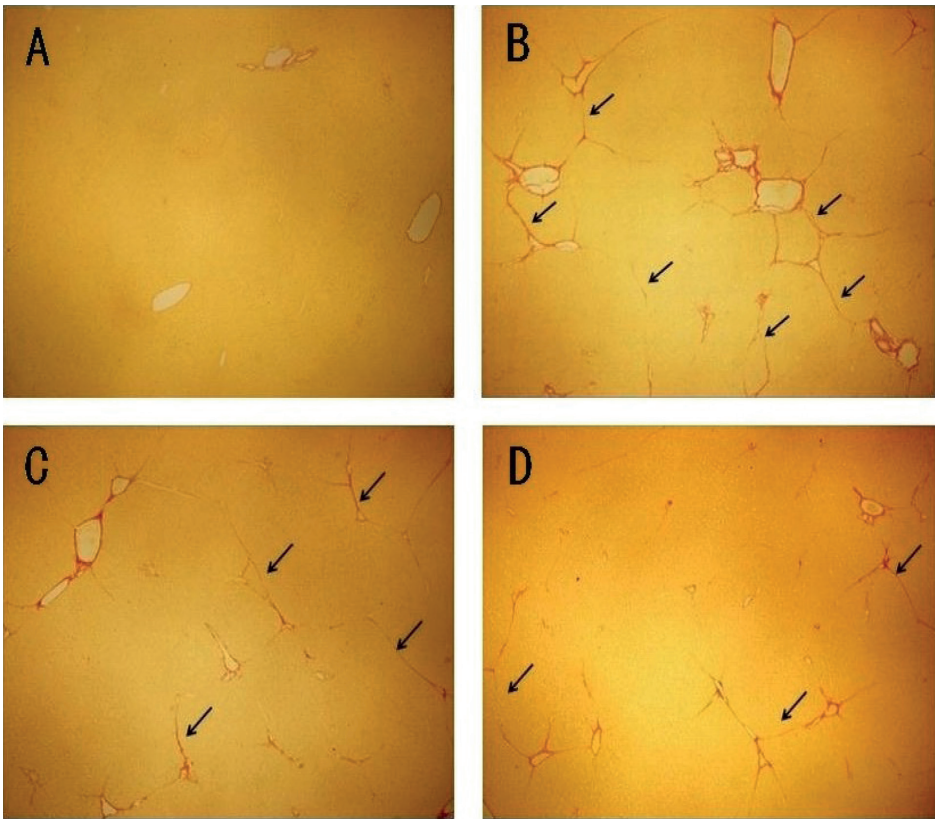

Figure 3. Effect of n-3 PUFA on cirrhosis change of the liver 7 days after PH. Sirius red staining of liver sections in each group. **A,** PH; **B,** LC+PH; **C,** LC+ n-3 PUFA+PH; **D,** LC+ n-3 PUFA*+PH. Arrows indicate positive staining. Original magnification x 100.

was observed around the portal regions in normal group (Figure 3A). However, positive staining was increased significantly in the LC+PH group 7 days after PH (Figure 3B). Positive staining was decreased significantly in the LC+n-3 PUFA+PH and LC+n-3 PUFA*+PH groups 7 days after PH (Figure 3C, Figure 3D). Masson's trichrome staining was observed in all groups. Positive staining (blue) was decreased significantly in the LC+n-3 PUFA+PH and LC+n-3 PUFA*+PH groups compared with the LC+PH group 7 days after PH (Figure 4). The immunohistochemical analysis of α-SMA was performed to assess the effect of n-3 PUFA on hepatic stellate cells activation during the cirrhosis development. No α-SMA positive cells could be found in PH group (Figure 5A). The activated hepatic stellate cells with expression of α-SMA grew significantly in the LC+PH group compared with LC+n-3 PUFA+PH and LC+n-3 PUFA*+PH groups 7 days after PH (Figure 5B, 5C, 5D).

N-3 PUFA Regulate Cytokines in Liver Cirrhosis

Compared with the LC+PH group, the levels of pro-inflammatory cytokines IL-6 was significantly down-regulated in the LC+n-3 PUFA*+PH group 1 day after PH (Figure 6A). The levels of anti-inflammatory cytokines IL-10 were significantly up-regulated in the LC+n-3 PUFA+PH and LC+n-3 PUFA*+PH group compared with the LC+PH 5 days after PH (Figure 6B).

N-3 PUFA Changes mRNA Expression Levels of TGF-β, MMP-9, and TIMP-1

TGF-β, MMP-9, and TIMP-1 mRNA expression levels were measured to assess the progression of cirrhosis changes in the liver by real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). In the LC+PH group, the TGF-β expression was significantly greater than that of the LC+n-3 PUFA*+PH group 7 days after PH ($p<0.05$) (Figure 7A). The MMP-9 level in the LC+n-3 PUFA+PH and LC+n-3 PUFA*+PH groups were significantly increased compared with the LC+PH group 7 days after PH ($p<0.05$) (Figure 7B). The TIMP-1 level in the LC+n-3 PUFA*+PH group was significantly decreased compared with the LC+ PH group 7 days after PH ($p<0.05$) (Figure 7C).

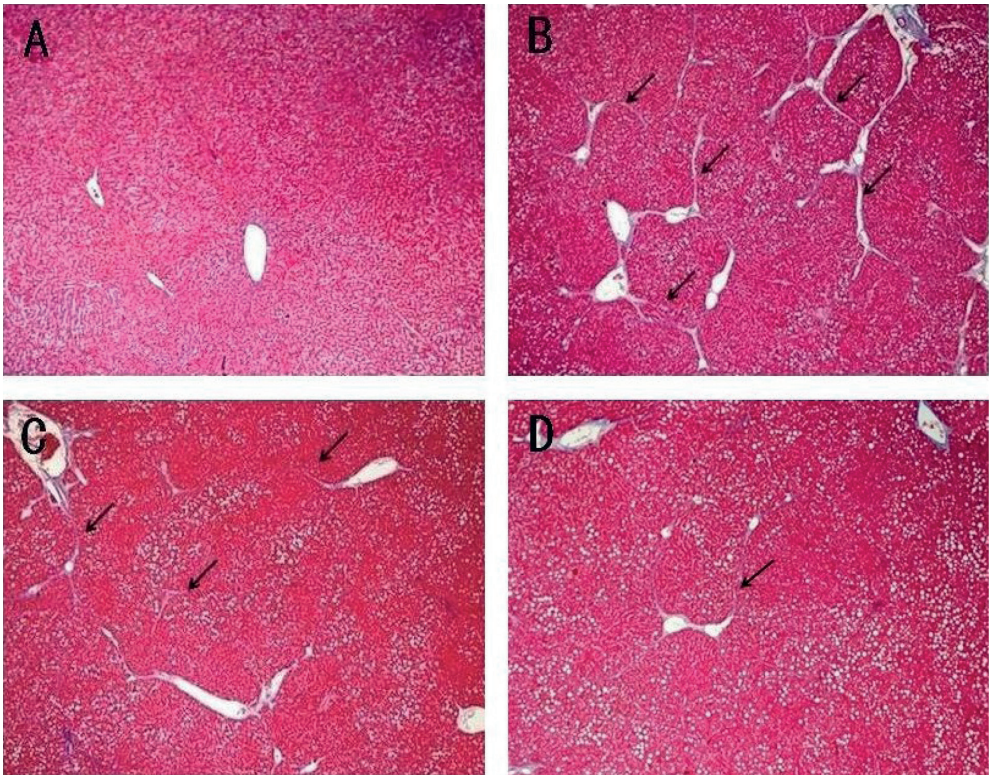

Figure 4. Effect of n-3 PUFA on Masson's trichrome staining of liver cirrhosis in all groups 7 days after PH. **A,** PH; **B,** LC+PH; **C,** LC+ n-3 PUFA+PH; **D,** LC+ n-3 PUFA*+PH. Arrows indicate positive staining. Original magnification x 100.

Discussion

Surgical resection is now the critical curative approach for HCC with liver cirrhosis[1]. However, liver regeneration of cirrhotic liver is significantly impaired, so how to promote liver regeneration and ameliorate liver cirrhosis after partial hepatectomy has been an urgent problem to be solved[5]. In this decade, reversibility of liver fibrosis in patients has been evidenced, which has inspired researchers to develop antifibrotic therapies[11]. In the previous study, some medical treatments were reported to be able to reverse the development of liver cirrhosis and improve liver regeneration e.g. thrombopoietin and antioxidants[12,13]. CCL_4 intoxication in rats was probably the most widely used for studying liver cirrhosis and CCl_4 administration to rats that causes fibrous change of the liver[14]. The present study was to investigate whether n-3 PUFA could reduce liver fibrosis and boost liver regeneration in cirrhosis induced by CCL_4. The results clearly indicated that besides boosting liver regeneration after 70% PH in rats, the administration of n-3 PUFA could also reduce the liver fibrosis.

Fatty acids, as essential nutrients, have wide ranges of biological functions and n-3 PUFA supplementation is also reported to involve in modifying the organic biochemical environment[15,16]. N-3 PUFA was widely used in clinical perioperative Total Parenteral Nutrition (TPN) and it was found to effectively protect liver, cardiovascular apparatus, and kidney[17-19]. In addition, n-3 PUFA was well known to regulate inflammatory cytokines in injury responses[9]. Hepatic cirrhosis is the result of the wound-healing response of the liver to repeated injury, and it is associated with an inflammatory response and a limited deposition of ECM[1]. This study found a protective effect of n-3 PUFA against liver fibrosis and enhanced liver regeneration after partial hepatectomy.

Qiu et al[20] focused on n-3 PUFA contribution to liver regeneration in the early period after PH. N-3 PUFA exhibited a strong anti-inflammatory effect, and this study presumed that the anti-inflammatory action could ameliorate acute liver failure after hepatectomy in cirrhotic liver; so, IL-6 and IL-10 were examined[9]. Jerin et al[21] suggested that the intensity of inflammation could be assessed by the ratio of IL-6/IL-10. In this study,

compared with LC+PH group, pro-inflammatory cytokine IL-6 significantly decreased as well as anti-inflammatory cytokine IL-10 significantly increased in LC+n-3 PUFA*+PH group. It was reported that N-3 PUFA[22] had no direct impact on the pathogenesis of normothermic ischemia reperfusion injury; whereas it could mediate tissue repair and liver regeneration. Giving CCl_4 to mice caused inflammatory change in the liver and up-reguated serum levels of AST and ALT. In this study, both ALT and AST levels were significantly lower in n-3 PUFA group 1 day and 3 days after the hepatectomy. Though the precise mechanism of interaction and balance between liver regeneration and fibrogenesis in the cirrhotic liver remains unclear[23], our results verified that n-3 PUFA induces liver regeneration even in cirrhotic liver.

CCl_4 caused hepatocytes necrosis and inflammation and led to liver fibrosis, which spread to link the vascular structures and finally developed cirrhosis. In the liver with persistent inflammation, hepatocytes were eventually substituted with abundant ECM, and HSC were activated or differentiated into myofibroblast-like cells[1,14]. Activated HSC migrated to the site of inflammation and secreted considerable amount of ECM. The accumulation of ECM proteins distorted the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes defines cirrhosis[1,23].

One of the reasons for promoting liver regeneration after PH of the cirrhotic liver was the antifibrotic effect of administered n-3 PUFA here. There has been no standard treatment for liver cirrhosis. However, some experimental investigations[24,25] revealed the targets to prevent cirrhosis progression in several years. Given that inflammation can precede and promote the progression of liver cirrhosis, the use of anti-inflammatory drugs has been proposed[23,24]. Suppressing the accumulation of the activated HSCs by modulating either their activation or promoting their apoptosis is the critical strategy. Drugs (e.g. thrombocytes, vitamin E, silymarin, and S-adenosyl-L-methionine) can suppress HSC activation, protect hepatocytes from suffering apoptosis and mitigate experimental liver cirrhosis[26-30]. Mehta el al[31] that antioxidants help to treat patients with alcohol-induced liver cirrhosis

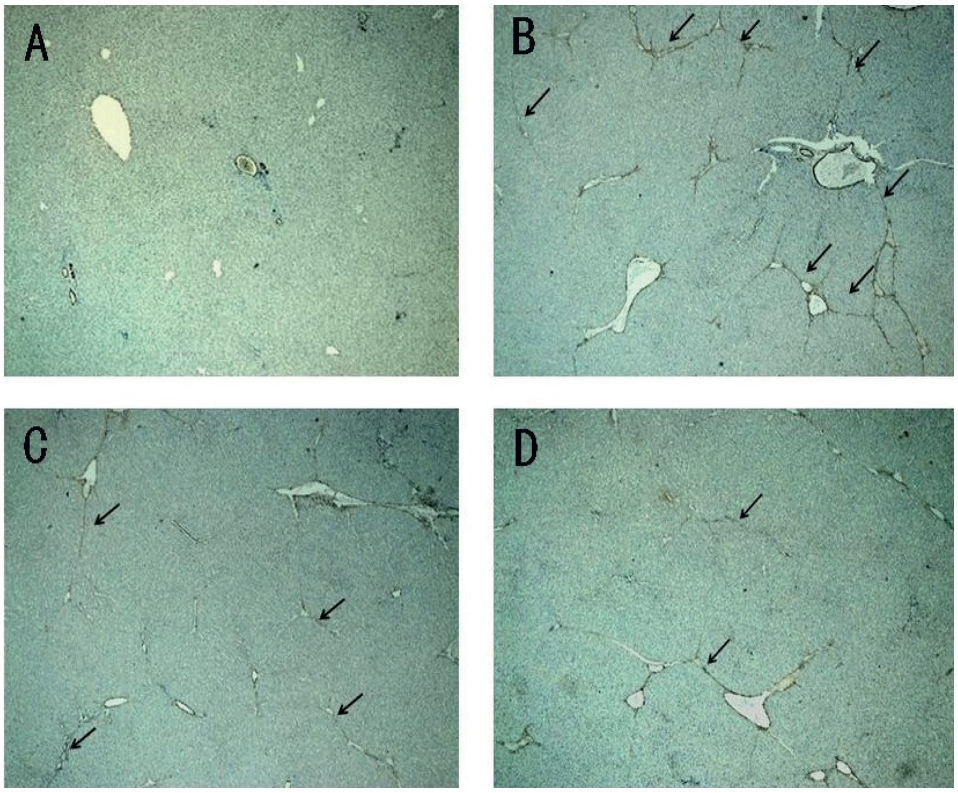

Figure 5. Effect of n-3 PUFA on activation of the hepatic stellate cells in the liver α-SMA staining of liver sections in all groups 7 days after PH. **A,** PH; **B,** LC+PH; **C,** LC+ n-3 PUFA+PH; **D,** LC+ n-3 PUFA*+PH. Arrows indicate α-SMA positive cells. Original magnification x 100.

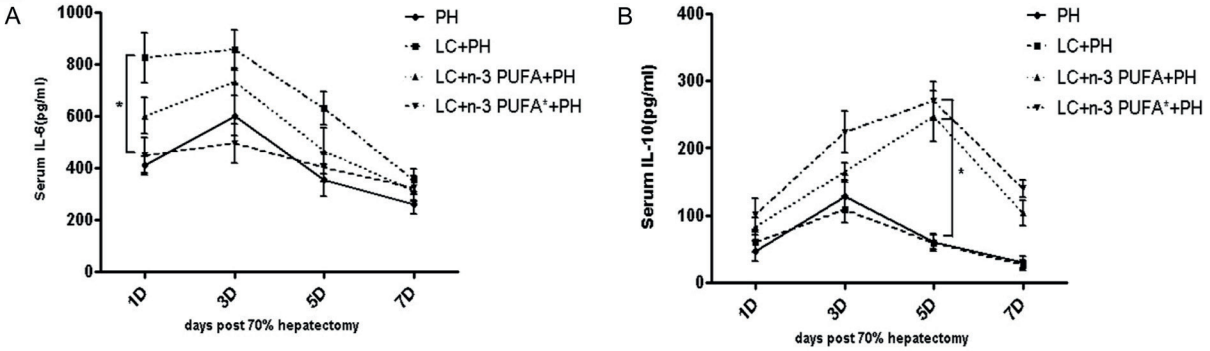

Figure 6. Changes of (***A***) cytokines interleukin IL-6 and (***B***) IL-10 in all groups after PH. $^{*}p<0.05$ *vs.* LC+PH group.

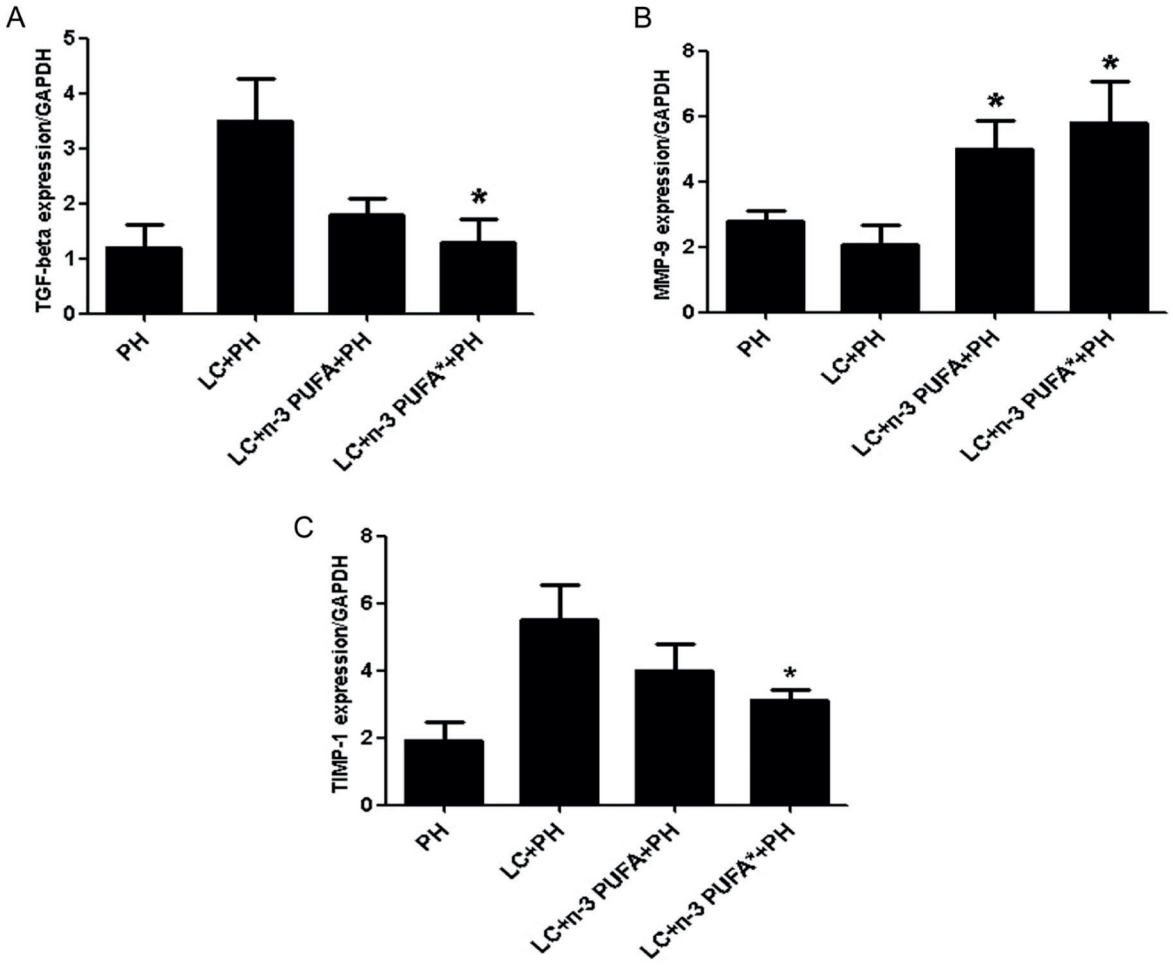

Figure 7. Messenger RNA expression level of TGF-β, MMP-9 and TIMP-1 in all groups 7 days after PH. **A,** TGF-β expression levels in the LC+ n-3 PUFA*+PH group were significantly suppressed compared to the LC+PH group (*$p<0.05$ *vs.* LC+PH group). **B,** MMP-9 expression in LC+ n-3 PUFA+PH and LC+ n-3 PUFA*+PH groups were significantly increased compared to the LC+PH group (*$p<0.05$ *vs.* LC+PH group). **C,** TIMP-1 expression in LC+ n-3 PUFA*+PH groups were significantly decreased compared to the LC+PH group (*$p<0.05$ *vs.* LC+PH group). Data are expressed as mean ±SD.

and nonalcoholic steatohepatitis (NASH). Damaged hepatocytes can release ROS and fibrogenic mediators and further induce the recruitment of white blood cells by inflammatory cells. Apoptosis of damaged hepatocytes can induce the fibrogenic actions of liver myofibroblasts[1,23]. N-3 PUFA has been reported[32] to modulate both nitric oxide synthase (NOS) activity and cyclooxygenase (COX) expression as antioxidants. NO was synthesized by three distinct NOS isoforms (namely neuronal, inducible, and endothelial NOS), and the expression levels of NOS could effectively halt liver fibrosis[33]. It disrupted TGF-β/MMP-9 synthesis and prevented scar formation in experiment liver cirrhosis[34]. TGF-β, which is primarily produced by Kupffer cells and HSC, significantly stimulates HSC to secrete ECM and inhibits ECM degradation by down-regulating MMP and promoting tissue inhibitors of matrix metalloproteinase (TIMP). The suppression of TGF-β expression may be attributed to the suppression of Kupffer cell activation or to the suppression of HSC activation is the critical process[35]. MMP-9 possesses proteolytic activity against type IV collagen, a major component of the basement membrane[36]. In this study, the expressions of TGF-β and TIMP-1 mRNA in the LC+PH group were significantly up-regulated compared with LC+ n-3 PUFA*+PH group 7 days after 70% PH. Once HSC become activated, expression of MMP increases, and during the process of HSC activation, HSC expression of TIMP is significantly increased[37]. Previous studies have reported that secreted MMP-9 is suppressed by HSC-derived TIMP1 and our results are consistent with this previous report. According to these results, n-3 PUFA may have special functions to reduce fibrous changes by a series of mechanisms. Further study is required to clarify this mechanism.

It was proposed that intravenously administration of n-3 PUFA will promote liver regeneration and slow progression of liver cirrhosis, and curative resection of advanced HCC could be achieved without liver failure. Recently, orally active n-3 PUFA has been clinically used to a large extent[38]. Accordingly, n-3 PUFA will be suitable for the sustainable improvement of liver function in liver cirrhosis.

Conclusions

The present experimental study clearly showed that n-3 PUFA mitigated the progression of liver cirrhosis and promoted liver regeneration after partial hepatectomy. According to the results here, n-3 PUFA contributed to the reduction of liver fibrosis, and a novel concept was developed for the clinical treatment of liver cirrhosis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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