Original Article
Diisopropylamine dichloroacetate alleviates liver fibrosis through inhibiting activation and proliferation of hepatic stellate cells

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Abstract: Our study was to elucidate the effects of DPDA on proliferation of LX2 cells *in vitro* and on liver fibrosis induced by carbon tetrachloride (CCl4) *in vivo*. Furthermore, the potential protective mechanism was also investigated. *In vitro*, the proliferation of LX2 cells was inhibited by DPDA, but the proliferation of LO2 cells was promoted when the concentration of DPDA was less than or equal to 1 mmol/l. Meanwhile, the expression of alpha-smooth muscle actin (α-SMA), Desmin, Transforming growth factor-beta 1 (TGF-β1) and Type I collagen were down regulated by DPDA dose-dependently. *In vivo* study, forty c57bl/6 male mice were divided into five groups, the control group (n=8), the CCl4 (4 w) group (n=8), the CCl4 (8 w) group (n=8), the CCl4 (12 w) group (n=8) and the DPDA+CCl4 group (n=8). The degree of liver fibrosis was assessed by HE and Masson's staining. Moreover, Immunohistochemistry and Western blot were used to detect the protein levels of α-SMA, Desmin, Ki67 and Type I collagen. HE and Masson stain showed that liver damage was alleviated in DPDA+CCl4 group compared to CCl4 (12 w) group. Meanwhile, the expression of α-SMA and Desmin was decreased. Furthermore, the level of Ki67 showed escalating trend. In conclusion, our study indicated that DPDA has the capacity to rescue liver fibrosis through inhibiting activated hepatic stellate cells. Therefore, it suggested that DPDA might be a potential medicine for liver fibrosis.

Keywords: Diisopropylamine dichloroacetate, liver fibrosis, hepatic stellate cells, TGF-β1, alpha-smooth muscle actin, type I collagen

Introduction

Liver fibrosis is a worldwide disease which arising from chronic liver injury caused by various etiological factors, including virus, alcohol, non-alcoholic fatty liver disease, liver metabolic disease and autoimmune disease [1, 2]. Liver fibrosis is a process characterized by increased accumulation of extracellular matrix (ECM) and excessive deposition of cross-linked collagens [3]. Currently, as far as we know, hepatic stellate cells (HSCs) activated by inflammatory cytokines are the core event in liver fibrosis [4-6]. Activated HSCs are capable of proliferation and migration, promoting the expression of alpha smooth muscle actin (α-SMA), matrix metalloproteinases (MMPs) and ECM [7, 8]. Transforming growth factor (TGF-β1) is considered to be the most important inflammatory cytokines, it can promote the transformation of

HSCs to muscle-fibroblasts as well as stimulate HSCs to produce a large amount of Type I collagen [8, 9]. With the progression of liver damages, fibrosis may lead to portal hypertension, cirrhosis and even hepatic carcinoma, which seriously affect the health of patients [10]. To date, effective treatments for liver fibrosis have not been developed yet. Thus, it is particularly important to develop effective anti-fibrotic agents.

Diisopropylamine dichloroacetate (DPDA) is the active component of pangamic acid and clinically available as a liver protectant [11]. DPDA is decomposed into diisopropylamine (DA) and dichloroacetate (DCA), promoting the methylation and regulating glucolipid metabolism [12, 13]. DPDA selectively inhibits pyruvate dehydrogenase kinase (PDK), resulting in significant restoration of pyruvate dehydrogenase (PDH)

activity, consequently regulates cell energy metabolism pathways [14, 15]. Our previous preliminary experiments showed that DCA could inhibit the proliferation of LX2 cells, but the function of DPDA on liver fibrosis has not been fully investigated.

In this study we elucidate the effect of DPDA on proliferation of LX2 cells *in vitro* and *in vivo* with liver fibrosis induced by carbon tetrachloride (CCl4). Furthermore, the potential protective mechanism was also explored.

Materials and methods

Cell culture

Human hepatic stellate cells LX2 cells and human normal liver cells LO2 cells were gifted by basic medical laboratory of Medical School of Nanjing University. LX2 cells and LO2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere with 5% CO_2 at 37°C. The medium was changed every 2-3 days.

To detect whether DPDA has effects on LX2 cells activation, different concentrations (0, 2, 4, 6, 8 mmol/l) DPDA were added *in vitro* for 48 hours, during which the medium was not changed. To the appointed time, cell pictures were taken in the inverted microscope photograph. Then, the cellular total protein was extracted and expression of α-SMA, Desmin, TGF-β1 and Type I collagen were detected by Western blot as described below, respectively.

Cell viability assay

Cell viability was detected by MTT assay. LX2 cells and LO2 cells (seeded at 5000-10000 cells per well in 96-well plates) were cultured overnight, treated with DPDA at the indicated concentration for 48 hours, and assessed for viability as follows (MTT added to each well (5 mg/ml, 20 μl per well), incubation for 4 h, solubilization of the reduced MTT dye in DMSO (150 μl per well), measurement of absorbance by spectrophomentry at 490 nm with a plate reader, and then using CmpoSyn software calculating the IC50 values.)

Cells protein extract

The LX2 were washed three times with cold PBS (4°C). Then cells were lysed with 180 equal volumes of RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% NaN_3, 1% sodium doecyl sulfate [SDS]) containing cocktail protease inhibitor (1:200; Sigma) on ice for 30 min, and sonicated four times for 5 s each. The cell lysates obtained after centrifugation (12,000 rpm at 4°C for 10 min) were subjected to Western blotting.

### Animal models and treatment

Six-week-old c57bl/6 male mice, weighing 18-22 grams, were used for our study. They were purchased from the Shanghai Laboratory Animal Center (Shanghai, China) and were housed in laboratory conditions (23°C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for 2 weeks prior to experimentation. Forty mice were randomly divided into five groups, such as the control group, the CCl4 (4 w) group, the CCl4 (8 w) group, the CCl4 (12 w) group, and the DPDA group. The model of liver fibrosis was induced by intraperitoneal injection with CCl4 (2 μl/g body weight dissolved 1:3 in corn oil, two times per week). The CCl4 (4 w) group, the CCl4 (8 w) group, the CCl4 (12 w) group, and the DPDA group were lasting for 4 w, 8 w, 12 w and 12 w, then were killed respectively. After 8 weeks injection with CCl4, mice were taken DPDA (15 mg/kg body weight, once a day) by intragastric administration in DPDA group. Meanwhile, mice of the CCl4 (12 w) group were only taken equal amount of PBS as contrast. All animal protocols were approved by institutional animal committee of Nanjing Drum Tower Hospital and experiments were performed in accordance with the guidelines for the care and use of experiment animals by the National Institutes of Health.

Histological examination

Liver tissues were embedded in paraffin and processed for hematoxylin-eosin (H&E) and Masson's trichrome staining. Five liver tissues were randomly selected from each group and which are from the same position of the liver. Furthermore, histopathological evaluation was exanimated by professional pathologists.

Immunohistochemistry

Formalin-fixed and paraffin-embedded liver sections with a thickness of 4 μm were dewaxed in xylene and graded alcohols, hydrated and
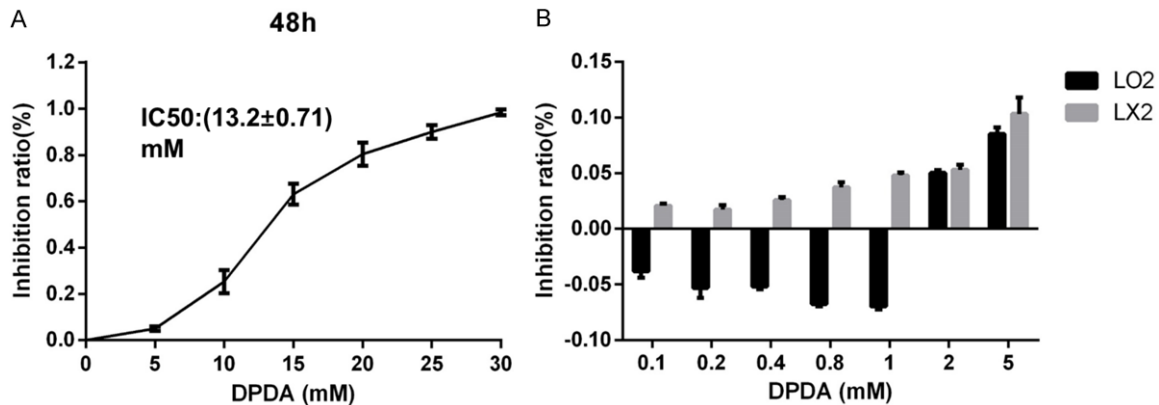
Figure 1. Effects of DPDA on proliferation of LX2 cells and LO2 cells. A. Curve of DPDA inhibiting proliferation of LX2 cells. B. Effects of DPDA in low does on proliferation of LX2 cells and LO2 cells. (This experiment repeated three times to get similar results).

washed in phosphate buffered saline (PBS). After pretreatment in a microwave oven, endogenous peroxidase was inhibited by 3% hydrogen peroxide in methanol for 20 min, followed by avidin-biotin blocking using a biotin-blocking kit (DAKO, Germany). Slides were then immunoassayed with the antibodies to α-SMA (1:500; Abcam), Desmin (1:300; Abcam) and Ki67 (1:400; Santa Cruz Biotechnology). A subsequent reaction was performed with biotin-free HRP enzyme labeled polymer from an En Vision plus detection system (DAKO, CA). Positive reactions were visualized with diaminobenzidine (DAB) solution followed by counter-staining with hematoxylin. A brown particle in nuclei was considered as positive labeling. Then liver section was observed under light microscopy and the staining intensity was assessed by professional pathologist.

Liver tissues protein extracting

Take 0.1 g liver tissue into grinding vessel, then pour into 1 ml volumes of RIPA lysis buffer containing cocktail protease inhibitor (described above) on ice, then fully gliding for 5 min. The tissue solution obtained after centrifugation (12,000 rpm at 4°C for 10 min) was subjected to Western blotting.

Western blotting

Protein extracted from the cells and tissues was quantified using the Coomassie plus protein assay reagent Pierce Chemical Co, IL, USA) and adjusted to an equal concentration for each sample before electrophoresis. The lysa-

tes were heated in Laemmli buffer at 95°C, resolved by 8% SDS-PAGE gel (Amresco, Ohio, USA),transferred to PVDF membrane (Roche, IN, USA), which was then incubated with 3% BSA in Tris Buffered Saline, followed by incubation with the antibodies α-SMA (1:1000; Abcam), Desmin (1:800; Abcam), TGF-β1 (1:1000; Santa Cruz Biotechnology) and Type I collagen (1:400; Abcam), and then with AP labeled secondary antibody (1:2000, Santa Cruz Biotechnology). The signals were determined using the enhanced chemiluminance assay (NBT/ BCIP Array, Huemei Bio).

Statistical analysis

Data were expressed as means ± standard deviation. Differences among groups were examined using a Kruskal-Wallis H test or a one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS version 19.0 for Windows (SPSS, Inc., Chicago, IL).

Results

Low-dose DPDA inhibits the proliferation of LX2 cells but promotes the proliferation of LO2 cells in vitro

48 hours after DPDA (concentration of 2.5, 5, 10, 15, 20, 25, 30 mmol/l) treated on LX2 cells, MTT showed that DPDA apparently inhibited the proliferation of LX2 cells with concentration dependence and the IC50 of DPDA on LX2 cells was (13.2±0.71) mmol/l (**Figure 1A**).
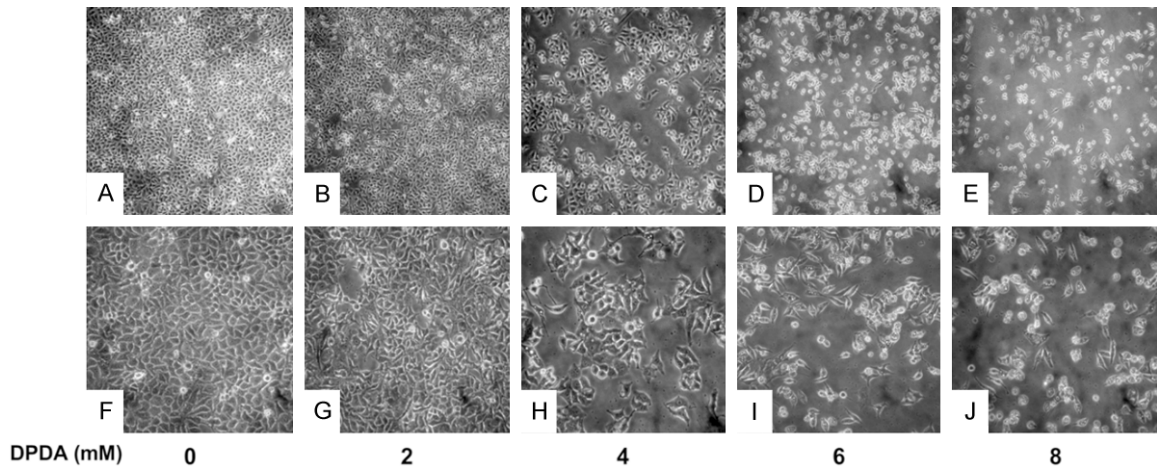
Figure 2. Effects of different concentrations of DPDA on the proliferation and morphology of LX2 cells. Treated LX2 cells with different concentrations of DPDA: 0, 2, 4, 6 and 8 mmol/l, the corresponding image was (A-E) under inverted microscope (40×) and (F-J) under inverted microscope (100×).

48 hours after low dose DPDA (0.1, 0.2, 0.4, 0.2, 1, 2, 5 mmol/l) acted on LX2 cells, MTT showed that low dose DPDA could inhibit the proliferation of LX2 cells. In addition, DPDA could inhibit the proliferation of LO2 cells when its concentration ≥ 1 mmol/l and an opposite function was displayed when the concentration of DPDA ≤ 1 mmol/l (**Figure 1B**).

48 hours after DPDA (concentration of 0, 2, 4, 6, 8 mmol/l) acted on LX2 cells, the cell counts and morphological changes were observed (**Figure 2**). With the increasing concentrations of DPDA, the number of LX2 cells was on the decline tendency within the same field of vision. Meanwhile, the cellular morphology changed from the spindle to a kind of circular gradually. In addition, numerous vacuoles were seen in cells vesicles. We speculated the mechanism of DPDA inhibiting the LX2 cells proliferation was that DPDA may decompose into small molecules, then through a certain way into cells, causing burst of cell death.

DPDA inhibits the activation of HSCs

To explore the role of DPDA in liver fibrosis, 48 hours after DPDA (concentration of 0, 2, 4, 6, 8 mmol/l) acted on LX2 cells, we used Western Blot to detect the expression of α-SMA, TGF-β1, Desmin and Type I collagen. With the increase of concentration of DPDA, the expression of α-SMA, TGF-β1, Desmin and Type I collagen are down-regulated (**Figure 3**). Results indicated

that DPDA may inhibit the activation of hepatic stellate cells to reverse liver fibrosis.

DPDA alleviates liver fibrosis in vivo

As shown in **Figure 4**, HE staining for the control group showed normal architecture, whereas from the CCl4 (4 w) group to the CCl4 (12 w) group, exhibiting the increasing fatty degeneration, necrosis, and inflammation of hepatocytes, even the CCl4 (12 w) group appeared cirrhosis false floccules. However, treatment with DPDA markedly improved the hepatic morphology and architecture with less pseudo-lobules and inflammatory cell infiltration compared with CCl4 (12 w) group (**Figure 4A**).

After Masson's staining, normal architecture was showed in the control group, while increasing extensive liver bridging fibrosis and substantial collagen deposition were exhibited in CCl4 groups and it became more and more obvious from 4 weeks to 12 weeks. However, in the DPDA group there was seldom fibrosis (**Figure 4B**).

In order to further evaluate the antifibrotic efficacy of DPDA, we examined the expression of proteins of the key fibrotic markers, including α-SMA and Desmin by Western Blot. We observed that the expression of α-SMA increased after the administration of CCl4. However, the DPDA group showed a markedly decreasing compared with CCl4 (12 w) group
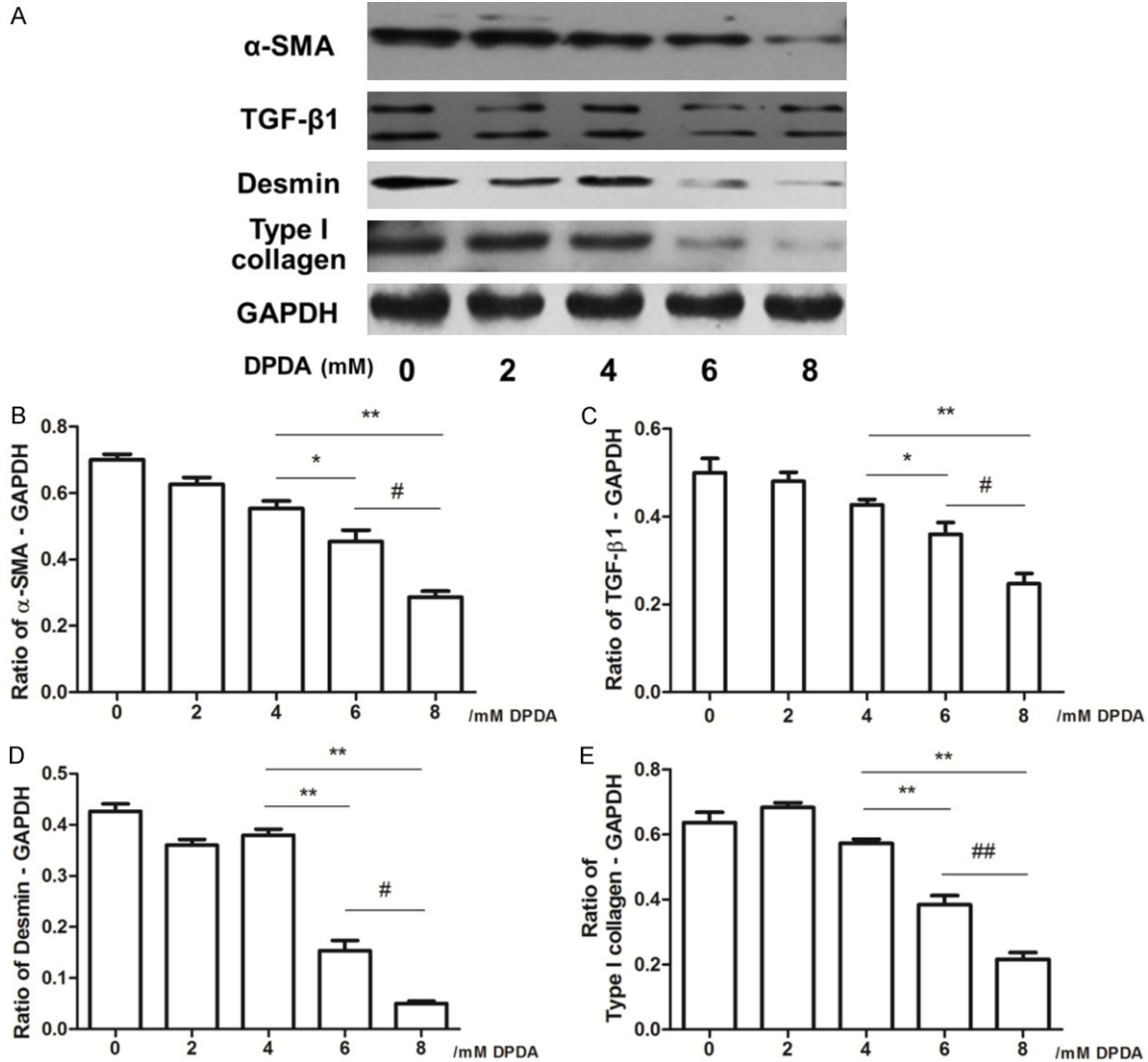
Figure 3. Expression of proteins related to liver fibrosis after DPDA acting on LX2 cells for 48 hours. The concentration of DPDA were 0, 2, 4, 6, 8 mmol/l orderly. A. Westren blotting was assessed to investigate protein level. B. Expression of α-SMA. C. Expression of TGF-β1. D. Expression of Desmin. E. Expression of Type I collagen. (TGF-β1 has two forms, unprocessed protein and mature protein, the molecular weight is 44 kDa and 12.5 kDa respectively. When they form the homologous dimer or heterogenous dimer, multiple bands can be seen, such as the bands on 40 kDa and 25 kDa side in my experiment).

(**Figure 4C**). But the expression of Desmin showed no significant difference (**Figure 4D**).

DPDA plays a role in liver protection through inhibiting the proliferation of hepatic stellate cells in vivo

As shown in **Figure 5**, after administration of CCl4, the degree of liver fibrosis was aggravating and appearing an increase in the number of fibroblasts. At the same time, compared with the CCl4 group, hepatocyte proliferation

increase and fibroblasts decrease in DPDA group. Furthermore, proteins were extracted from the control group, CCl4 (4 w) group, CCl4 (8 w) group CCl4 (12 w) group and DPDA group, then detect the expression of α-SMA, Desmin and Type I collagen with Western blot. Results reflected that with the administration of CCl4, the expression of α-SMA and Type I collagen increased gradually. Compared with CCl4 (12 w) group, the expression of these proteins in DPDA group decreased (**Figure 6**). However, the expression of Desmin showed no significant
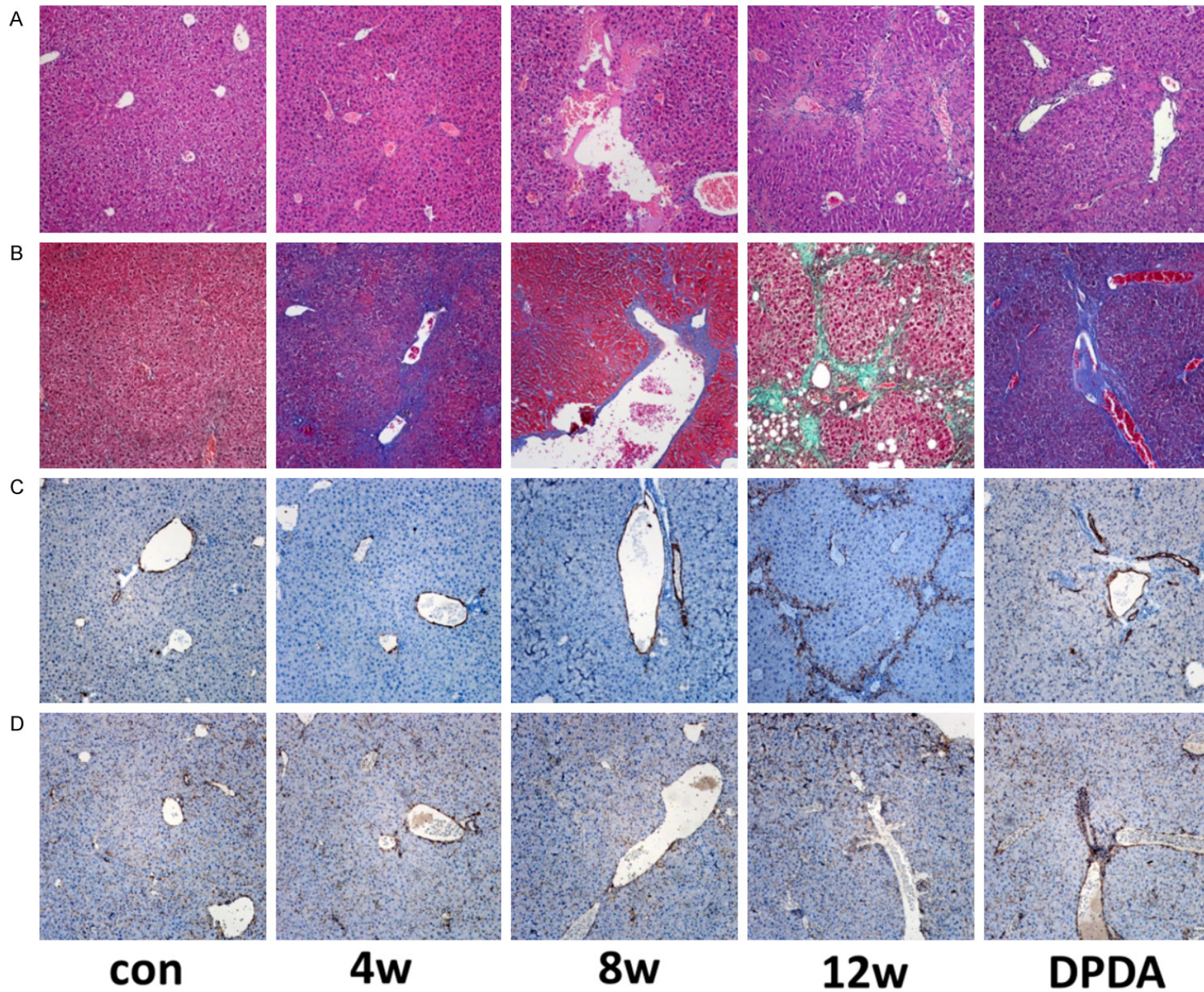
Figure 4. Effects of DPDA on liver histological changes by CCl4 in mice. A. Liver sections were stained with hematoxylin and eosin (HE) in mice treated with the methods, such as vehicle, CCl4 (4 w), CCl4 (8 w), CCl4 (12 w) and CCl4+DPDA group respectively. B. Masson's staining was used to detect the accumulated collagen in liver sections from vehicle, CCl4 (4 w), CCl4 (8 w), CCl4 (12 w) and CCl4+DPDA group respectively. C. Expression of α-SMA was used as the key fibrotic markers to detect the antifibrosis of DPDA. D. Expression of Desmin was used as the key fibrotic markers to detect the antifibrosis of DPDA (100×).
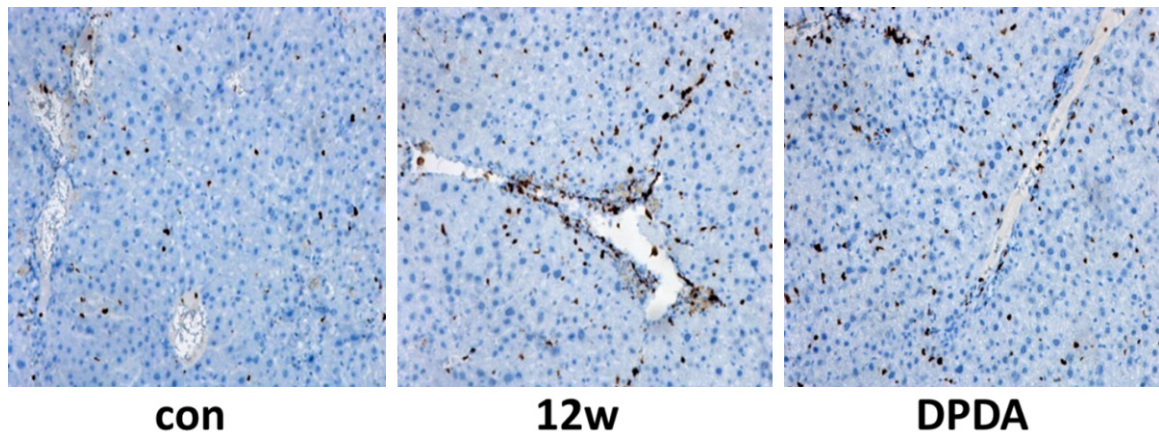
Figure 5. Expression of Ki-67 was used to detect the cells proliferation of control, CCl4 (12 w) and CCl4+DPDA group respectively (100×).
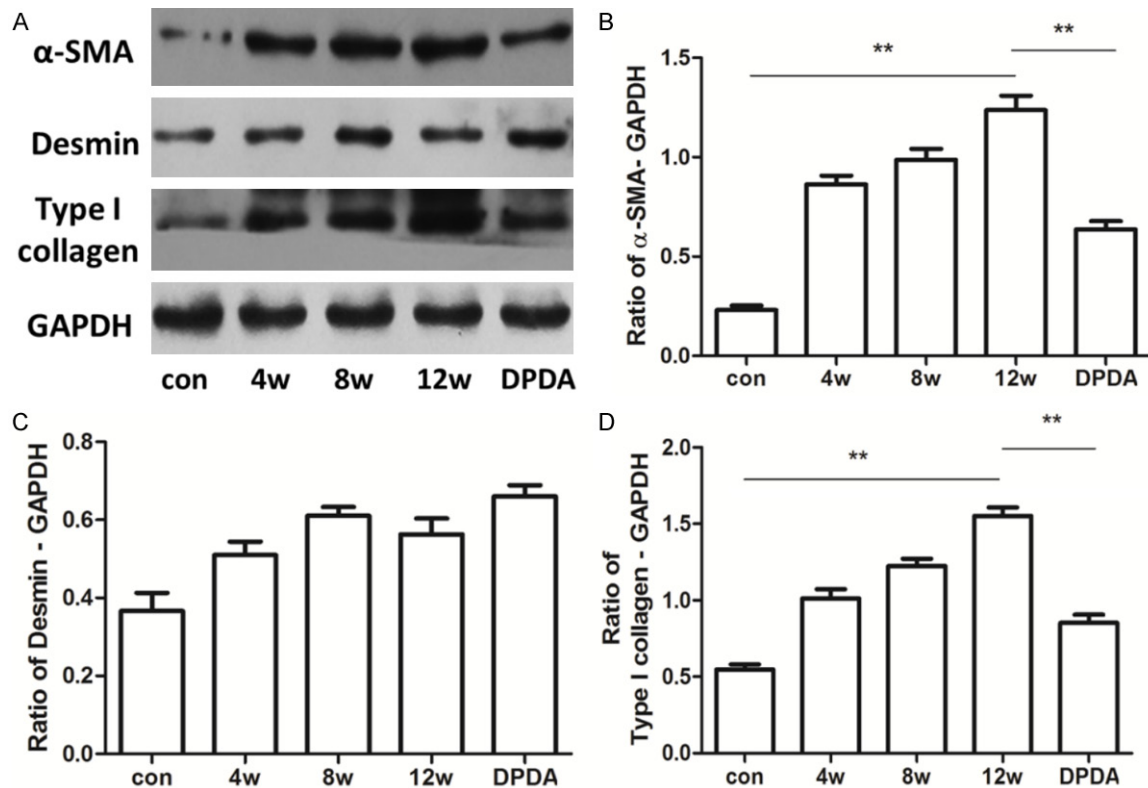
Figure 6. Effects of DPDA on alleviating liver fibrosis. A. Western blotting was assessed to investigate protein level. B. Expression of α-SMA. C. Expression of Desmin. D. Expression of Type I collagen.

importance. It is concluded that DPDA plays a role in liver protection through promoting liver cell proliferation and inhibiting the activation of hepatic stellate cells.

Discussion

Liver fibrosis is a process that various harmful factors acting on liver, resulting in liver tissue damage, and then excessive proliferation and abnormal fiber connective tissue deposition [16, 17]. Professor Rogking proposed that human liver fibrosis can be reversed, and experts in liver area have no objection to this view [18, 19]. Although a large number of drugs have been reported having the antifibrosis function in recent years, but most of the researches are still *in vitro* or animal experimental stage, the effective and safe study in human body has not yet been fully confirmed. The proliferation and activation of HSC to myofibroblast-like cells and deposition of collagen are its key characteristic in liver fibrosis [20, 21]. In the progression of liver fibrosis, various cytokines especially TGF-β1 were hold the balance [22], which was accordant with our study. In our research, a liver fibrosis model was successfully established through intraperitoneal injection with CCl4 in mice, as evidenced by histological evaluation and upregulated expression of TGF-β1 and α-SMA.

DPDA, a vitamin B15 active ingredient [23], is a kind of protective liver medicine, having the clinical curative effects [24]. As an old medicine, DPDA has recently attracted renewed attention. Studies show that DPDA interventions lead to necrosis, inflammation, and fibrosis of tumor tissue in a mouse subcutaneous transplantation breast tumor model, exerting potential therapeutic effects [15]. Also, DPDA can treat liver damage caused by a variety of causes. However, whether DPDA has effects on liver fibrogenesis and its potential mechanism is still unknown. In our study, results showed that DPDA has an antifibrotic efficacy to the pathogenesis of liver fibrosis. *In vitro*, results showed that with the increasing concentrations of DPDA, LX2 cells numbers were on the decline tendency. Moreover, the expression of α-SMA, TGF-β1, Desmin and Type I collagen are downregulated. We conclude that DPDA can inhibit the proliferation of hepatic stellate cells.

In addition, in the liver fibrosis model study presenting the increased expression of α-SMA induced by CCl4 and histological change improved in the DPDA groups, suggesting that DPDA can protect the mice against liver fibrosis induced by CCl4 to some extent, which was accordance with our study *in vitro*.

In conclusion, we show that administration of DPDA suppressed the expression of α-SMA, TGF-β1, Desmin and Type I collagen in cultured LX2 cells and in CCl4 induced liver fibrosis model of mice. Therefore, via inhibiting the proliferation and activation of HSC, DPDA alleviates the progression of liver fibrosis.

Acknowledgements

Disclosure of conflict of interest

None.

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