Stem bark of *Fraxinus rhynchophylla* ameliorates the severity of pancreatic fibrosis by regulating the TGF-β/Smad signaling pathway

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ABSTRACT

Chronic pancreatitis (CP) is a pathological fibroinflammatory syndrome of the pancreas. Currently, there are no therapeutic agents available for treating CP-associated pancreatic fibrosis. *Fraxinus rhynchophylla* (FR) reportedly exhibits anti-inflammatory, antioxidative and antitumor activities. Although FR possesses numerous properties associated with the regulation of diverse diseases, the effects of FR on CP remain unknown. Herein, we examined the effects of FR on CP. For CP induction, mice were intraperitoneally administered cerulein (50 µg/kg) 6 times a day, 4 days per week for 3 weeks. FR extract (100 or 400 mg/kg) or saline (control group) was intraperitoneally injected 1 hour before the first cerulein injection. After 3 weeks, the pancreas was harvested for histological analysis. In addition, pancreatic stellate cells (PSCs) were isolated to examine the antifibrogenic effects and regulatory mechanisms of FR. Administration of FR significantly inhibited histological damage in the pancreas, increased pancreatic acinar cell survival, decreased PSC activation and collagen deposition, and decreased pro-inflammatory cytokines. Moreover, FR treatment inhibited the expression of fibrotic mediators, such as α-smooth muscle actin (α-SMA), collagen, fibronectin 1, and decreased pro-inflammatory cytokines in isolated PSCs stimulated with transforming growth factor (TGF)-β. Furthermore, FR treatment suppressed the phosphorylation of Smad 2/3 but not of Smad 1/5 in TGF-β-stimulated PSCs. Collectively, these results suggest that FR ameliorates pancreatic fibrosis by inhibiting PSC activation during CP.

Significance of this study

What is already known about this subject?

⇒ Chronic pancreatitis (CP) is a progressive, irreversible disease process characterized by inflammation and fibrosis.
⇒ Cerulein is the most common and suitable for models of CP in mice.
⇒ Pancreatic stellate cells (PSCs) are central cells of pancreatic fibrosis and Smad 2/3 phosphorylated by fibrotic stimulation.

What are the new findings?

⇒ *Fraxinus rhynchophylla* (FR) inhibits acinar cell death and infiltration of inflammatiory cells during CP.
⇒ FR inhibits the activation of PSC, thereby reducing the production of extracellular matrix, a key feature of fibrosis.
⇒ FR inhibits phosphorylation of Smad 2/3 in PSCs.

How might these results change the focus of research or clinical practice?

⇒ The advantage of natural product-derived therapeutics is high safety, and FR can be considered as a potential therapeutic agent for CP.
⇒ FR, which inhibits pancreatic fibrosis, may also be effective in other models such as liver fibrosis.

INTRODUCTION

Chronic pancreatitis (CP) is an irreversible, progressive disease characterized by chronic inflammation, glandular necrosis, and fibrosis[1–3] and can result in exocrine and endocrine pancreatic dysfunctions.[4] Clinical manifestations include persistent or recurrent abdominal pain, indigestion, malnutrition, anorexia, diabetes, and related complications, such as the formation of pseudocysts, which negatively impact the patients' quality of life.[5] CP is considered an important risk factor for pancreatic cancer.[6] Pancreatic fibrosis is considered a critical event in the progression of CP and is deemed the key therapeutic target.[7–9] To date, the treatment of CP has focused on supportive therapies to reduce symptoms. However, no effective strategies have been established for treating pancreatic fibrosis. Therefore, identifying and developing pharmacological agents that can control pancreatic damage, inflammation, and fibrosis remain critical.

Activated pancreatic stellate cells (PSCs) are the main effector cells in the process of fibrosis, which is the main pathological feature of CP.[10] Typically, PSCs are in a quiescent state and

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regulate extracellular matrix (ECM) production. Notably, transforming growth factor (TGF)-β is the representative stimulatory cytokine found in PSCs and produced by inflammatory mediators.[11] TGF-β is one of the most effective regulatory cytokines mediating the fibrotic response.[12] TGF-β can induce the differentiation of PSCs into myofibroblasts, which can secrete various ECM proteins, such as type I collagen and fibronectin.[13–15] In addition, it has been reported that Smads are functionally dynamic in PSCs.[16] TGF-β promotes the formation of polymers of Smad2 and Smad3 and then enters the nucleus to regulate the transcription of target genes, thereby promoting pancreatic fibrosis.[17] Therefore, blocking the nuclear translocation of the TGF-β signal and Smad2/3 phosphorylation could suppress PSC activation, thus affording a therapeutic effect in CP.

Fraxinus rhynchophylla (FR), commonly known as ash tree, belongs to the family Oleaceae.[18] This deciduous tree grows widely in Korea and China. The bark of this tree has been used as an antibacterial, analgesic, and anti-inflammatory agent and is known as the traditional Chinese herbal drug 'Qinpi' in the Chinese Pharmacopeia. However, it remains unclear whether FR can reduce pancreatic fibrosis during CP.[19] Therefore, we aimed to determine the anti-inflammatory and antifibrotic effects of FR in cerulein-induced CP mice. Accordingly, we examined histological variations in the pancreas, as well as typical fibrosis-related features such as PSC activation, collagen deposition, and production of cytokines. In addition, the effects of FR on TGF-β-induced PSC activation and collagen production, and production of cytokines were examined using primary mouse PSCs.

MATERIALS AND METHODS

Materials

Cerulein was purchased from Bachem Laboratories (Bubendorf, Switzerland). Hematoxylin, eosin, xylene, and ethanol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Easy-Blue Total RNA extraction kit and radioimmunoprecipitation assay (RIPA) buffer were purchased from iNtRON Biotechnology (Seongnam, KyungKiDo, South Korea). Antibodies against phospho-specific Smad 2/3 (cat. no. 8828S), Smad 1/5 (cat. no. 9516S), Smad 2/3 (cat. no. 3102S), and Smad 1/5 (cat. no. 9517S) were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Antibodies against alpha-smooth muscle actin (α-SMA; cat. no. sc-32251) and β-actin (cat. no. sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Plant materials

FR was purchased from Kwangmyungdang Medicinal Herbs (Nam-gu, Ulsan, South Korea). The identity of the herb was confirmed at Wonkwang University. The FR extract was prepared by decocting dried FR (100 g) with boiling distilled water (1 L) for approximately 2 hours and 30 min. The water extract was frozen at −80°C and then freeze-dried to produce a powder (9.3 g). The extraction yield was 9.3%. The powder was extracted using distilled water and filtered. The final FR concentration was 100 mg/mL. The filtrates were stored at 4°C until further use.

Animals

C57BL/6 mice (6–8 weeks of age, weighing 15–20 g, free of murine-specific pathogens) were purchased from Samtako BioKorea (Osan, KyungKiDo, South Korea). All animals were bred and housed in standard shoebox cages in a climate-controlled room with an ambient temperature of 23°C±2°C and a 12-hour light-dark cycle for 7 days. The animals were fed standard laboratory chow and allowed water ad libitum under standard conditions with air filtration. All studies were designed to generate groups of equal size using randomization and blinded analysis.

Experimental design

CP was induced by intraperitoneally administering cerulein (50 μg/kg, n=6 per group for three experiments, total=18) 6 times at intervals of 1 hour, 4 times per week, for a total of 3 weeks as previously described.[20] In the pretreatment groups, FR (100 or 400 mg/kg, n=6 per group for three experiments, total=18) or saline (control group, n=6 per group for three experiments, total=18) was administered by intraperitoneal injection 1 hour before the first cerulein injection, once daily, 4 times a week, for a total of 3 weeks. Mice were sacrificed 24 hours after the last cerulein injection via CO_2 asphyxiation, and the pancreases were harvested for further analysis. All experiments were independently performed 3 times.

Histological analysis

Briefly, harvested pancreatic tissues were fixed in 4% paraformaldehyde for 24 hours, dehydrated in different grades of ethyl alcohol, and embedded in paraffin. The paraffin-embedded sections (4 μm thick) were stained with H&E for histological examination using light microscopy. Morphological changes were scored for pancreatic tissue edema, inflammation, and fibrosis, as previously reported.[20] Then, pancreatic tissue sections representing a minimum of 100 fields were examined for each sample and scored on a scale of 0–3 (0, normal; 3, severe).

Immunofluorescence

For immunofluorescence analysis of α-SMA, pancreatic tissues were embedded in optimal temperature cutting medium (Tissue Tek). Serial sections (9 μm thick) were cut using a cryostat microtome and fixed in 100% methanol at −20°C for 5 min. The slides were permeabilized with 0.1% Triton X-100 for 15 min at room temperature (RT), followed by washing with phosphate-buffered saline containing 0.05% Tween-20 (PBST). The slides were then incubated in blocking buffer containing 5% goat serum in PBST for 1 hour at RT, followed by incubation with primary antibodies: α-SMA (Santa Cruz Biotechnology, cat. no. sc-32251) overnight at 4°C. The tissues were then rinsed 3 times for 5 min in PBST and incubated with the following secondary antibodies: Alexa Fluor 594 goat anti-mouse (1:2000 dilution in 5% goat serum in PBST, Invitrogen; Thermo Fisher Scientific, cat. no. A32742) at RT for 2 hours. Finally, the nuclei were visualized with DAPI (1:2000 dilution in 5% goat serum in PBST, Vector Laboratories, Burlingame, California, USA, cat. no. H-1200) for 5 min at RT. Stained slides were visualized using a confocal laser microscope (Olympus, Tokyo, Japan).

Quantitative reverse transcription-PCR

Total RNA was extracted from pancreatic tissues or PSCs using an Easy-Blue Total RNA extraction kit, according to the manufacturer's instructions. RNA was reverse transcribed to complementary DNA using a ReverTra Ace quantitative reverse transcription-PCR (qRT-PCR) Kit (Toyobo, Osaka, Japan), and qPCR was performed using TaqMan and THUNDERBIRD Probe qPCR Mix, according to the manufacturer's instructions (Toyobo). For each sample, triplicate test reactions and a control reaction without reverse transcriptase were performed to measure the expression of the gene of interest and control variations in the reactions. All data were normalized to that of the housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT). Forward, reverse, and probe oligonucleotide primers for multiplex real-time TaqMan PCR were purchased from ABI (Applied Biosystems, Foster City, California, USA), including HPRT (Mm03024075_m1), Acta2 (Mm01546133_m1), fibronectin 1 (Fn1) (Mm01256734_m1), collagen I (Mm00483888_m1), collagen III (Mm00802300_m1), interleukin (IL)-1β (Mm00434228_m1), IL-6 (Mm00446191_m1), and tumor necrosis factor (TNF)-α (Mm00443258_m1). The data were analyzed using CFX Maestro software (V.2.0; Bio-Rad Laboratories, Hercules, California, USA). The $2^{-\Delta\Delta Cq}$ method was used to determine the relative mRNA expression levels.[21]

Masson's trichrome staining

The extent of collagen deposition in the pancreatic tissue was determined using a Masson's trichrome (MT) staining kit (Polysciences, Pennsylvania, USA) according to the manufacturer's instructions.

Briefly, pancreatic tissue sections (4 μm thick), after deparaffinization and rehydration, were stained with Bouin's solution for 15 min, followed by nuclear staining with Weigert's hematoxylin for 10 min. The tissues were then stained with Biebrich scarlet-acid fuchsin solution for 5 min. Slides were then treated with phosphotungstic/phosphomolybdic acid solution for 10 min and placed in aniline blue solution for 5 min. Finally, slides were placed in a 1% acetic acid solution for 1 min. Collagen fibers were stained blue. Relative intensity was measured using light microscopy software (Olympus).

Isolation of mouse PSCs

PSCs were isolated from C57BL/6 mice by digesting the pancreatic tissue and Nycodenz (Sigma-Aldrich; Merck KGaA) density gradient centrifugation, as described previously.[13] Briefly, freshly isolated mouse PSCs were cultured in Dulbecco's Modified Eagle Medium/high glucose, supplemented with 10% fetal bovine serum and 2% penicillin-streptomycin at 37°C with 5% CO_2. Cells in passages 2–4 were employed.

Western blot analysis

Proteins were extracted from PSCs using lysis buffer (1% cocktail of protease inhibitor and 1% phosphatase inhibitor in 1×RIPA buffer). The protein concentration was determined using a bicinchoninic acid protein assay kit. Equal amounts of protein samples (20 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). The membranes were blocked with 5% skim milk in PBST at RT for 1 hour. Next, the membranes were incubated with primary antibodies against pSmad 2/3 and pSmad 1/5 (1:1000 dilution in PBST) overnight at 4°C, washed 3 times with PBST, and then incubated with horseradish peroxidase (HRP)-conjugated goat antirabbit secondary antibody (1:5000 dilution in PBST; Novus Biologicals, Centennial, Colorado, USA) for 1 hour at RT. Protein bands were developed using chemiluminescence with an EZ-Western Lumi Pico Kit (DoGenBio, Seoul, Korea), according to the manufacturer's recommended protocol. Protein bands were captured using Ez-capture ST (AE-9160PH, ATTO, Tokyo, Japan), and quantitative analysis was performed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

Data values are expressed as the mean±standard error of the mean (SEM). Significance was evaluated using a two-way analysis of variance (ANOVA) with time and dose as parameters. Significant ANOVA tests underwent post hoc analysis using the Duncan test for multiple comparisons among groups. Statistical significance was set at $p<0.05$.

RESULTS

Effects of FR extract on pancreatic damage in cerulein-induced CP

First, the histological architecture of the pancreas was investigated to examine the effects of FR on the development of CP. CP mice exhibited histological changes in the pancreas, including severe acinar architectural damage (edema and fibrosis) and inflammatory cell infiltration (inflammation). Conversely, FR treatment reduced pancreatic damage and inflammatory cell infiltration following cerulein-induced CP (figure 1).

Effects of FR extract on PSC activation during CP

Following activation, PSCs transform into a myofibroblast-like phenotype and exhibit increased α-SMA accumulation.[22] Thus, we measured pancreatic α-SMA expression as a marker of activated PSCs using immunofluorescence staining. Pancreatic tissues from CP mice revealed a significant increase in α-SMA-positive cells, whereas FR-treated mice showed a decrease in α-SMA-positive cells (figure 2A,B). In addition, treatment with FR reduced the mRNA expression of alpha-actin-2 (Acta-2) (figure 2C).

Effects of FR on ECM production during CP

Tissue fibrosis is one of the main characteristics of CP. Accordingly, we performed MT staining to examine collagen deposition. In CP mice, MT staining revealed a marked increase in pancreatic collagen deposition; FR treatment decreased collagen deposition (figure 3A,B). Furthermore, the enhanced mRNA expression of fibrosis-related genes (Fn1, collagen I, and collagen III) was reduced following FR treatment (figure 3C-E).

Effects of FR extract on production of pro-inflammatory cytokines during CP

Cytokines such as IL-1β, IL-6, and TNF-α are important in the progression of pancreatic fibrogenesis in CP, affecting

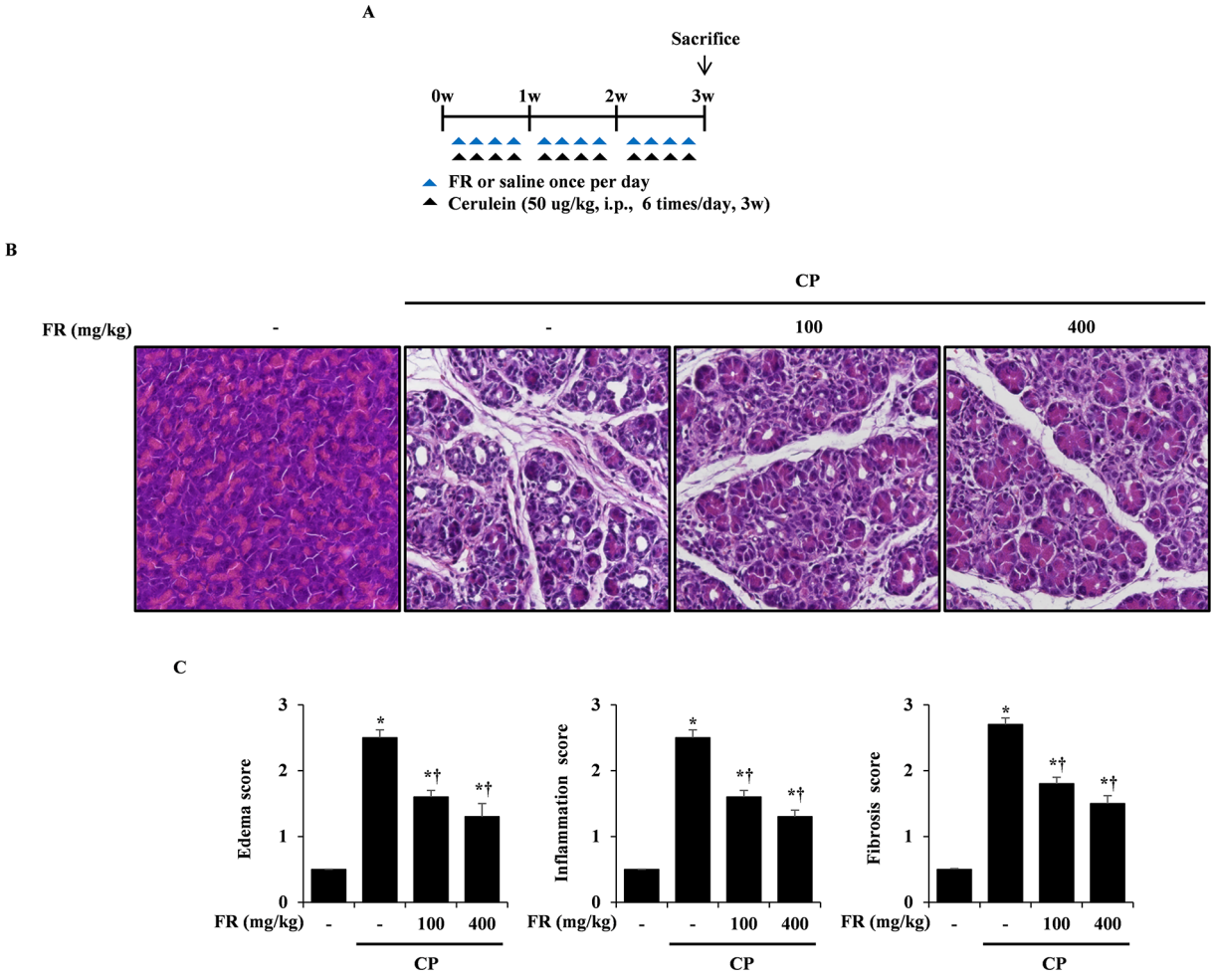

Figure 1 Effect of *Fraxinus rhynchophylla* (FR) extract on pancreatic damage in cerulein-induced chronic pancreatitis (CP). (A) CP was induced by administering six intraperitoneal injections of cerulein (50 µg/kg) at 1 hour intervals, 4 times a week, for 3 weeks. FR (100 or 400 mg/kg) or saline was intraperitoneally administered 1 hour before the first cerulein injection, once daily, 4 times a week, for 3 weeks. Mice were sacrificed 3 weeks after initiating the cerulein injection. (B) Representative H&E-stained pancreas section (200× magnification). (C) Histological sections of the pancreas were scored from 0 (normal) to 3 (severe) for edema, inflammation, and fibrosis. Data are presented as mean±standard error of the mean (SEM) for six mice in each group. Results are representative of three experiments. *P<0.05 vs saline alone. †P<0.05 vs CP.

PSCs.[23–25] Thus, we investigated whether FR reduces the production of pro-inflammatory cytokines in the pancreas during CP. Our results showed that CP mice significantly increased mRNA levels of IL-1β, IL-6, and TNF-α, while FR treatment could reduce significant downregulation of these genes (figure 4).

Effects of FR extract on the TGF-β/Smad signaling pathway in activated PSCs

The TGF-β/Smad signaling pathway is responsible for the development of pancreatic fibrosis in CP.[26] TGF-β stimulates its downstream Smad proteins via the transcription of ECM components.[26 27] Therefore, we cultured PSCs to investigate the antifibrotic mechanism of FR. As shown in figure 5A–E, TGF-β-stimulated PSCs exhibited increased mRNA expression levels of fibrotic genes, including Acta2, Fn1, collagen I, and collagen III, and also showed an increased mRNA expression levels pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α. However, FR treatment significantly suppressed the increased mRNA expression of TGF-β. Furthermore, the protein of phosphorylated Smad 2/3 was substantially increased in TGF-β-induced PSCs. However, FR treatment markedly suppressed phosphorylated Smad2/3 (figure 5F).

DISCUSSION

In the present study, we aimed to determine the protective effect of FR extract in a CP mouse model. Our findings revealed that FR treatment decreased acinar cell death, inflammatory cell infiltration, and collagen deposition in the pancreas. In addition, FR treatment inhibited PSC activation by reducing Acta-2 and collagen levels. Accordingly, our results suggest that FR ameliorates the severity of CP in the pancreas by inhibiting PSC activation.

CP is a persistent inflammatory pancreatic disease characterized by irreversible morphological changes, permanent loss of function, and pancreatic fibrosis. Previous reports indicate that 61% of patients with CP require at least one

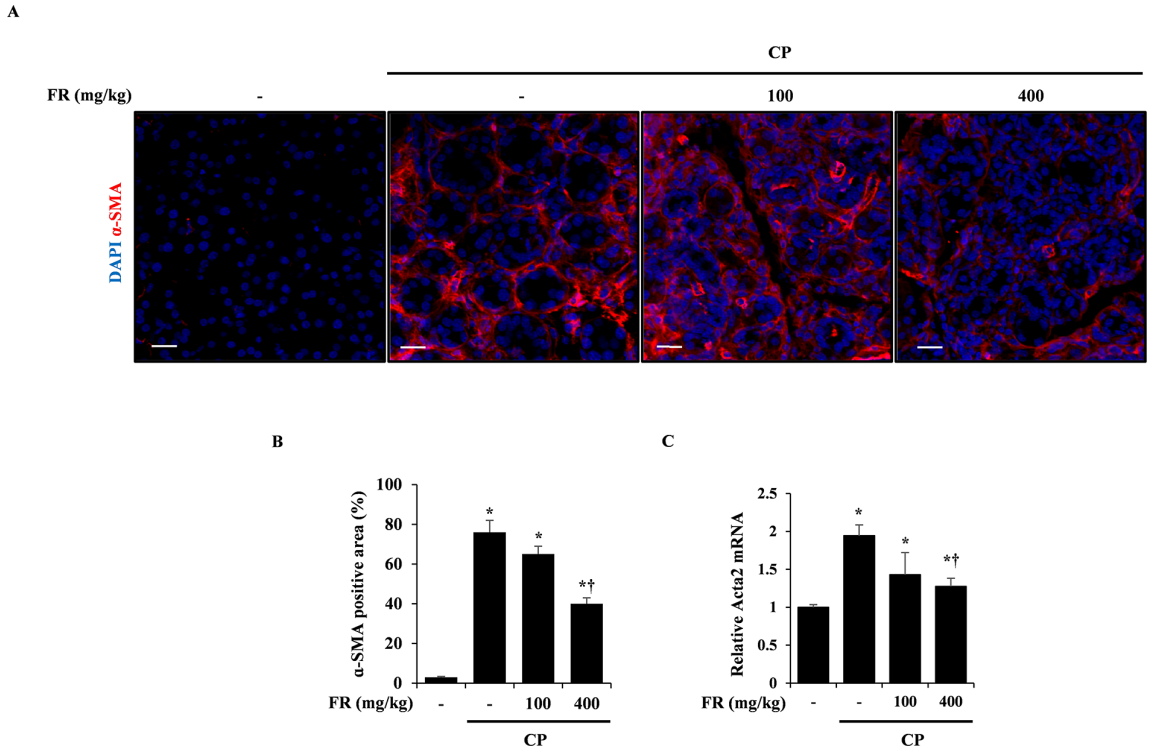

Figure 2 Effect of *Fraxinus rhynchophylla* (FR) extract on the activation of pancreatic stellate cells (PSCs) in cerulein-induced chronic pancreatitis (CP). (A) Confocal images of immunofluorescent staining of alpha-smooth muscle actin (α-SMA). α-SMA is stained red; nuclei are stained blue with 4,6-diamidino-2-phenylindole (DAPI). Scale bar=20 µm. (B) Relative intensity of α-SMA. (C) Pancreatic mRNA level of alpha-actin-2 (Acta-2) was determined using quantitative reverse transcription-PCR (qRT-PCR). Data are presented as mean±standard error of the mean (SEM) for six mice in each group. Results are representative of three experiments. *P<0.05 vs saline alone. †P<0.05 vs CP.

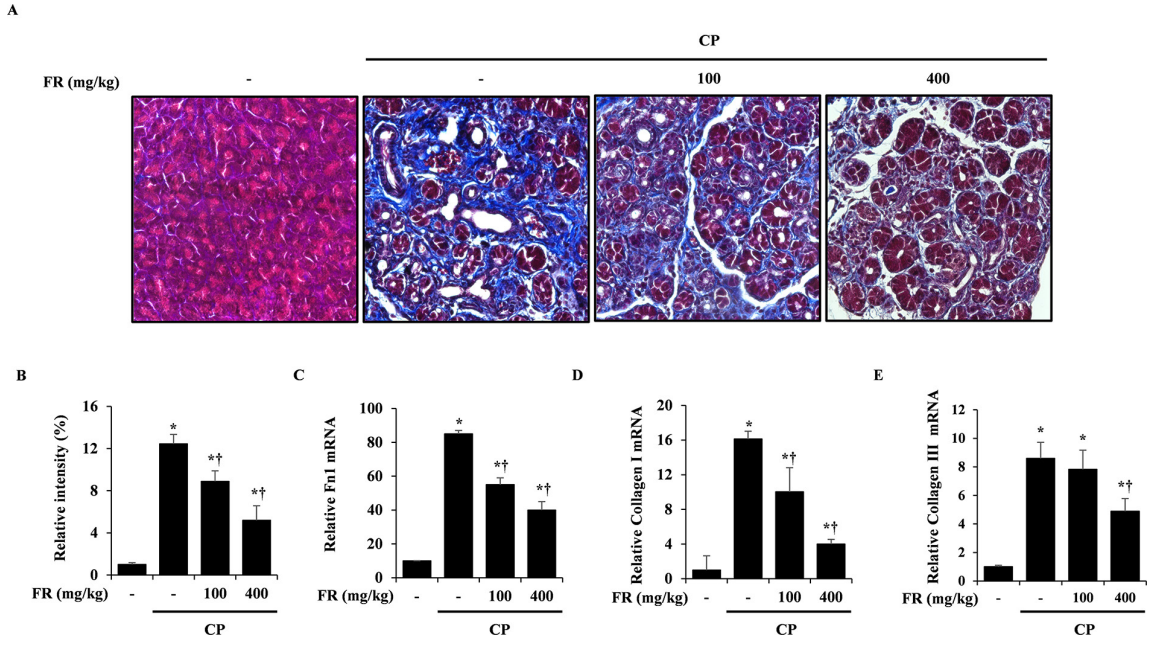

Figure 3 Effect of *Fraxinus rhynchophylla* (FR) extract on collagen deposition during chronic pancreatitis (CP). (A) Collagen deposition in the pancreas was examined using Masson's trichrome (MT) staining (200× magnification). (B) Relative intensity of MT staining. Pancreatic mRNA levels of (C) fibronectin 1 (Fn1), (D) collagen I, (E) collagen III were determined using quantitative reverse transcription-PCR (qRT-PCR).

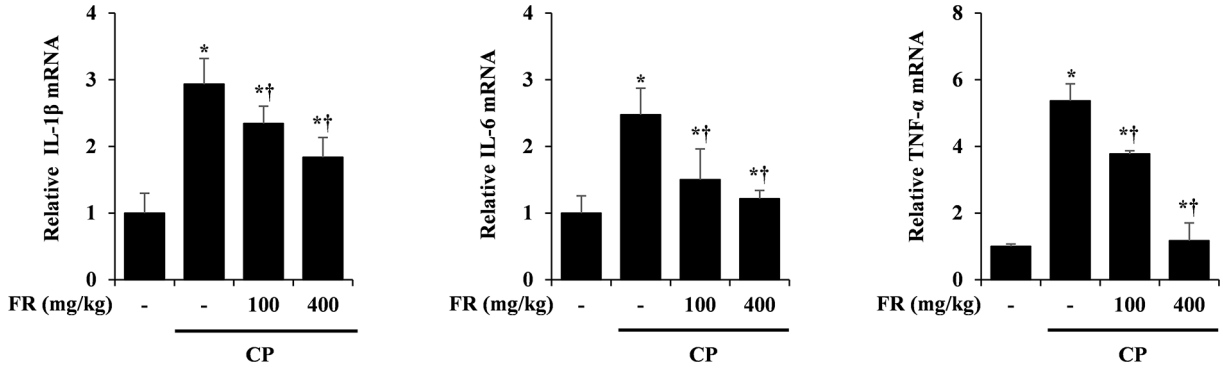

Figure 4 Effect of *Fraxinus rhynchophylla* (FR) extract on cytokines production during chronic pancreatitis (CP). mRNA expression levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were measured using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Data are presented as mean±standard error of the mean (SEM) for six mice in each group. Results are representative of three experiments. *P<0.05 vs saline alone. †P<0.05 vs CP.

endoscopic intervention, and 31% necessitate surgical intervention as part of their treatment strategy.[28 29] Nevertheless, the 30-day readmission rate of patients with CP, owing to a wide range of related complications, is reportedly 26.7%, which in turn exposes patients to the risk of opioid use disorder during long-term drug therapy.[30–32] Therefore, interest in natural herbs with low cost and high efficacy is gaining momentum, and as side effects are limited, these compounds can be developed as an alternative for long-term treatment.[33 34] In addition, the biological function of FR has been well demonstrated; in particular, FR reportedly inhibits liver fibrosis, suggesting that FR can potentially regulate CP.[19]

Although CP is caused by multiple etiologies, including alcohol and smoking, the main feature ultimately involves altering the healthy pancreas into fibrotic tissue. The initial event that induces pancreatic fibrosis is damage to one or all of the various tissue compartments or cell types of the pancreas, resulting in cell atrophy and the release of cytokines that recruit inflammatory cells.[35] Our results showed that the administration of FR reduced cell atrophy, inflammatory cell infiltration, and fibrosis (figure 1). The release of cytokines and growth factors such as IL-1β, IL-6, TNF-α, and TGF-β1 play an important role in the development of CP inflammation and fibrosis. When the pancreas is damaged, infiltrated inflammatory cells accelerate the activation and

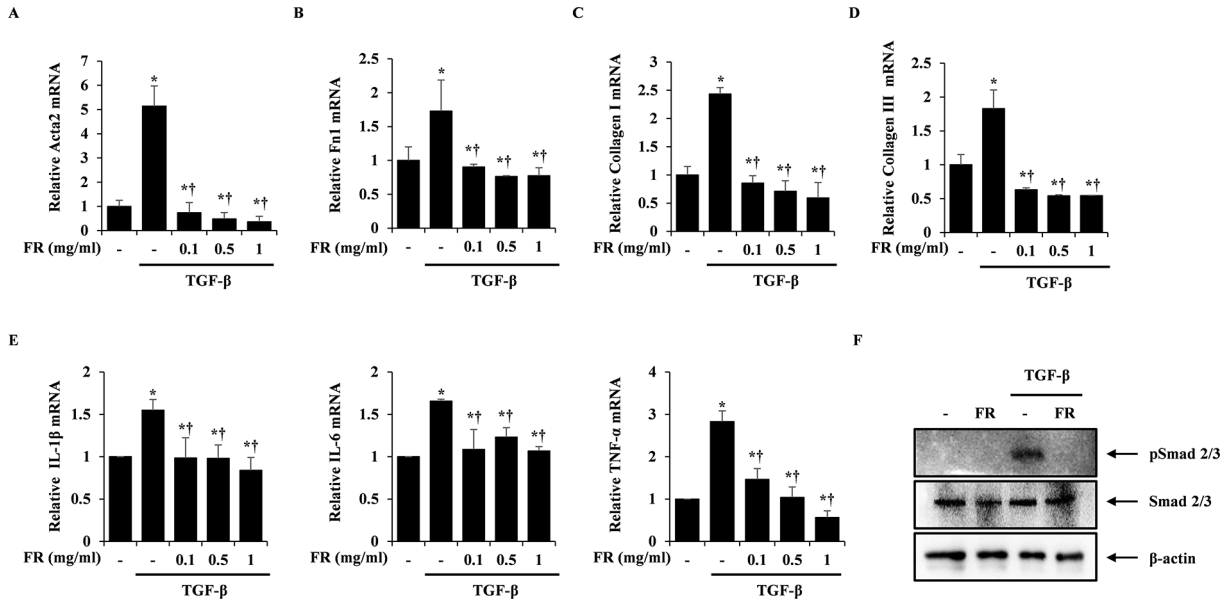

Figure 5 Effect of *Fraxinus rhynchophylla* (FR) extract on transforming growth factor (TGF)-β-induced pancreatic stellate cell (PSC) activation and the TGF-β/Smad signaling pathway in isolated PSCs. Mouse PSCs were pretreated with FR (0.1, 0.5, or 1 mg/mL) for 1 hour and then stimulated with TGF-β (0.5 ng/mL) for 24 hours. mRNA expression levels of (A) alpha-actin-2 (Acta-2), (B) fibronectin 1 (Fn1), (C) collagen I, (D) collagen III, (E) interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were determined using quantitative reverse transcription-PCR (qRT-PCR). Mouse PSCs were pretreated with FR (1 mg/mL) for 1 hour and then stimulated with TGF-β (0.5 ng/mL) for 30 min. (F) Phosphorylation levels of Smad 2/3 and Smad 1/5 were analyzed by western blot analysis and Smad2/3, Smad 1/5, and β-actin were used as loading controls. Data are presented as mean±standard error of the mean (SEM) for six mice in each group. Results are representative of three experiments. *P<0.05 vs saline alone. †P<0.05 vs TGF-β.

proliferation of PSCs, inducing their transformation into a myofibroblast phenotype, thus producing ECM components such as collagen and fibronectin.[12 23 36] Therefore, the regulation of PSCs in CP is crucial for treating CP. In the present study, α-SMA-positive areas were increased, collagen and fibronectin were upregulated, and pro-inflammatory cytokines were also upregulated during CP. FR treatment suppressed the severity of fibrosis and inflammation in the pancreas, suggesting that the beneficial effects of FR in CP are mediated via inhibition of PSC activation (figures 2–4).

At damaged sites, TGF-β1 is associated with excessive inflammation and fibrosis development.[37] Numerous studies have previously reported that overexpression of TGF-β causes fibrosis during diseases, and its role in CP has also been well-established.[25 38] In addition, inhibition of TGF-β1 reduces ECM production in cerulein-induced pancreatitis.[39] Moreover, TGF-β expression has been detected in human CP tissues, indicating that TGF-β plays a pivotal role in pancreatic fibrosis.[40] Therefore, we used TGF-β1 as a PSC stimulator. Additionally, in hepatic stellate cells (HSCs), an inflammatory cytokine was upregulated by stimulated with TGF-β.[41] The physiological role of PSCs show similarities to HSCs and similar role in response to injury stimuli. Therefore, we measured TGF-β-induced inflammatory cytokines on PSCs. As shown in figure 5, compared with TGF-β alone, FR significantly inhibited the expression of α-SMA, Fn1, collagen, and cytokines in activated PSCs, suggesting that FR inhibits TGF-β-induced fibrosis in PSCs. Furthermore, a previous report has demonstrated that the Smad 2/3 signaling pathway is crucial for TGF-β-mediated PSC activation.[16] In the present study, we examined the effects of FR on the TGF-β/Smad signaling pathway. Our results revealed that FR treatment suppressed the phosphorylation of Smad2/3 induced by TGF-β1 in PSCs, suggesting that FR downregulates PSC activation by decreasing Smad2/3 phosphorylation (figure 5E). Collectively, our findings reveal that FR inhibits pancreatic fibrosis and suggest that its beneficial effects are mediated via the TGF-β/Smad pathway.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval All experiments were performed according to protocols approved by the Animal Care Committee of Wonkwang University (WKU 20-54). This study does not involve human participants.

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Data availability statement Data are available on reasonable request.

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