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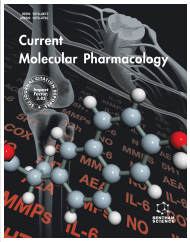

RESEARCH ARTICLE

Calpain Inhibitor Calpeptin Improves Pancreatic Fibrosis in Mice with Chronic Pancreatitis by Inhibiting the Activation of Pancreatic Stellate Cells

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Abstract:

Background:

Pancreatic fibrosis is a hallmark feature of chronic pancreatitis (CP), resulting in persistent damage to the pancreas. The sustained activation of pancreatic stellate cells (PSCs) plays a pivotal role in the progression of pancreatic fibrosis and is a major source of extracellular matrix (ECM) deposition during pancreatic injury.

Methods:

Calpain is a calcium-independent lysosomal neutral cysteine endopeptidase and was found to be correlated to various fibrotic diseases. Studies have revealed that calpeptin, a calpain inhibitor, can improve the fibrosis process of multiple organs. This study investigated the effect of the calpain inhibitor, calpeptin, on fibrosis in experimental CP and activation of cultured PSCs in mice. CP was induced in mice by repeated injections of cerulein for four weeks *in vivo*, and the activation process of mouse PSCs was isolated and cultured *in vitro*. Then, the inhibitory effect of calpeptin on pancreatic fibrosis was confirmed based on the histological damage of CP, the expression of α-smooth muscle actin (α-SMA) and collagen-Iα1(Col1α1), and the decrease in mRNA levels of calpain-1 and calpain-2.

Results:

In addition, it was revealed that calpeptin can inhibit the activation process of PSCs and induce significant PSCs apoptosis by downregulating the expression of calpain-1, calpain-2 and TGF-β1, and the expression and phosphorylation of smad3 *in vitro*.

Conclusion:

These results suggest that the calpain inhibitor, calpeptin, plays a key role in the regulation of PSC activation by inhibiting the TGF-β1/smad3 signaling pathway, which supports the potential of calpeptin as an inhibitor of pancreatic fibrosis in mice by interfering with calpain.

Keywords: Calpeptin, Chronic pancreatitis, Pancreatic fibrosis, Pancreatic stellate cells, Calpain, TGF-β1/smad3 signaling pathway.

Article History	Received: January 20, 2023	Revised: October 01, 2023	Accepted: October 10, 2023

1. INTRODUCTION

Chronic pancreatitis (CP) refers to the persistent inflammatory response of pancreatic tissues caused by various etiologies, leading to the progressive destruction of pancreatic parenchyma and, ultimately, pancreatic endocrine insufficiency. The typical pathological manifestations are pancreatic parenchymal atrophy and pancreatic fibrosis. The formation of pancreatic fibrosis is mainly due to the increase in synthesis and deposition of the collagen-based extracellular matrix (ECM), and research evidence has indicated that pancreatic stellate cells (PSCs) are important cells involved in pancreatic fibrosis [1 - 3].

There is clear evidence that supports transforming growth factor (TGF)-β1 as a key regulator of PSCs and its sustained activation during the development of chronic pancreatitis.

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TGF-β1 induces the expression of α-smooth muscle actin (SMA) and ECM proteins in PSCs and activates PSCs to become fibroblasts with a pro-fibrotic phenotype [4]. However, there is presently no specific treatment for CP. Therefore, some research on pancreatic fibrosis would provide a new possible approach to treating CP.

Calpain is a calcium-independent lysosomal neutral cysteine endopeptidase. At the same time, it was found that calpain is correlated to various fibrotic diseases and that inhibiting calpain through calpeptin can improve the fibrosis process of some organs. It was reported that calpain inhibitors can prevent bleomycin-induced lung fibrosis in mice [5, 6]. For the mechanism of calpain in pulmonary fibrosis, another study revealed that TGF-β1 increases the calpain activity and collagen-Iα1(Col1α1) synthesis in lung fibroblasts, thereby contributing to the pathogenesis of pulmonary fibrosis [7]. Consistent with the study conducted by Letavernier, a study revealed that calpain can induce myocardial fibrosis, in part, through the activation of TGF-β [8]. Furthermore, some studies reported that calpain plays a key role in collagen synthesis in pulmonary vascular remodeling [9, 10], and calpain can mediate pulmonary vascular remodeling through the secretion of the TGF-β1 pathway [10].

In recent years, it has been considered that PSCs are important cells involved in pancreatic fibrosis [11, 12]. Research evidence has shown that activated PSCs promote pancreatic fibrosis progression [13] and pancreatic cancer progression [14, 15] and play a key role in the repair of acute pancreatitis and pancreatic regeneration [16]. The phenotypic and functional changes of PSCs during activation are caused by the changes in signaling pathways in stellate cells. However, there is a lack of research on the role of the calpain gene in the process of PSC activation. Therefore, the present study determined whether calpeptin has a therapeutic effect on CP and pancreatic fibrosis. At the same time, the expression of calpain-1 and calpain-2 and the effect of calpeptin on the TGF-β1/smad3 signaling pathway were observed in the PSCs activation process.

2. MATERIALS AND METHODS

2.1. Ethics Statement

All animal-related procedures were approved by the Animal Care and Use Committee of Shanghai Shanghai General Hospital, Shanghai Jiaotong University School of Medicine (Approval no. 2019AW059). Maintain mice at 22°C under a 12 h light-dark cycle with ad libitum access to water and standard laboratory chow and allow the mice to acclimatize for at least one week. The environment was maintained at 30-70% relative humidity. Mice with cerulein-induced pancreatitis were monitored as described above.

2.2. Materials and rReagents

The cerulein and calpeptin (Cal) were purchased from MedChemExpress (Shanghai, China). The antibodies against calpain-1, calpain-2, TGF-β1 and TGFR2 were purchased from Proteintech (Shanghai, China). The α-SMA was purchased from Santa Cruz (CA, USA). The antibodies against Col1α1, vimentin, GAPDH, Smad3 and phospho-Smad3 were obtained from Cell Signaling Technology (Danfoss, USA).

2.3. Animals and Experimental Design

Male C57BL/6J mice were purchased from Shanghai Jihui Experimental Animal Co. Ltd. (Shanghai, China). These mice were randomly distributed (n=40, weight: 20 ±2g) into four groups, with 10 animals in each group. Then, CP and pancreatic fibrosis were induced in mice by repeated episodes of acute pancreatitis [12]. Acute pancreatitis was induced by hourly (six times) intraperitoneal (*i.p.*) injections of cerulein at 50 μg/kg of mice body weight, while the control group received equivalent volumes of normal saline (NS) [17]. Mice in the CP group received six *i.p.* injections of cerulein (MedChemExpress, China) three days a week at a dose of 50 μg/kg. The calpeptin group received calpeptin (MedChemExpress, China) dissolved in 0.1 mL of 5% DMSO in 0.1 mL of normal saline (Fig. **1**). Mice in the CP+Calpeptin2W (CP+Cal2W) group initially received cerulein for three days a week from the first day. Then, the (CP+Cal2W) group was started at 0.04 mg calpeptin per day from the first day of the third week until the end of the experiment for a total of two weeks, simulating the treatment process. Mice in the CP+Calpeptin4W (CP+Cal4W) group received cerulein injections for three days a week and 0.04 mg calpeptin per day from the first day of the experimental period for a total of four weeks, simulating the preventive course of treatment. At the end of the 4-week study period, these mice were sacrificed under 3% sodium pentobarbital anesthesia (Fig. **1**). For each case, the pancreas was rapidly excised, the sections were fixed in 4% paraformaldehyde buffered with normal saline (NS) overnight at 4°C, and they were either embedded in paraffin or immediately frozen in at 80°C. The remaining sections were quickly grounded in liquid nitrogen and frozen at -80°C for further study. Blood samples were kept at room temperature for two hours and centrifuged at 3,000 g for 15 minutes at 4°C. Then, the serum was stored at -80°C.

2.4. Serum Amylase and Lipase Analysis

The serum amylase and lipase levels were measured by enzyme kinetic chemistry using commercial kits, according to the Roche/Hitachi Modular Analysis System (Roche, Mannheim, Germany) protocol.

2.5. Histological Examination

Mouse pancreatic histology was examined by hematoxylin-eosin (H&E) and Masson trichromatic staining. A portion of pancreatic tissue was immobilized in 4% phosphate-buffered formaldehyde for 24 h, dehydrated by gradient ethanol series, and embedded in paraffin blocks. Pancreatic sections (5 μm) were dewaxed in xylene, hydrated with an upgraded ethanol series, and stained with H&E and Masson. Then, a semi-quantitative grading score was used to assess the severity of CP: graded glandular atrophy (0-3), intralobular, interlobular, and periductal fibrosis (0-3), and inflammatory monocyte infiltration (0-3) [15].

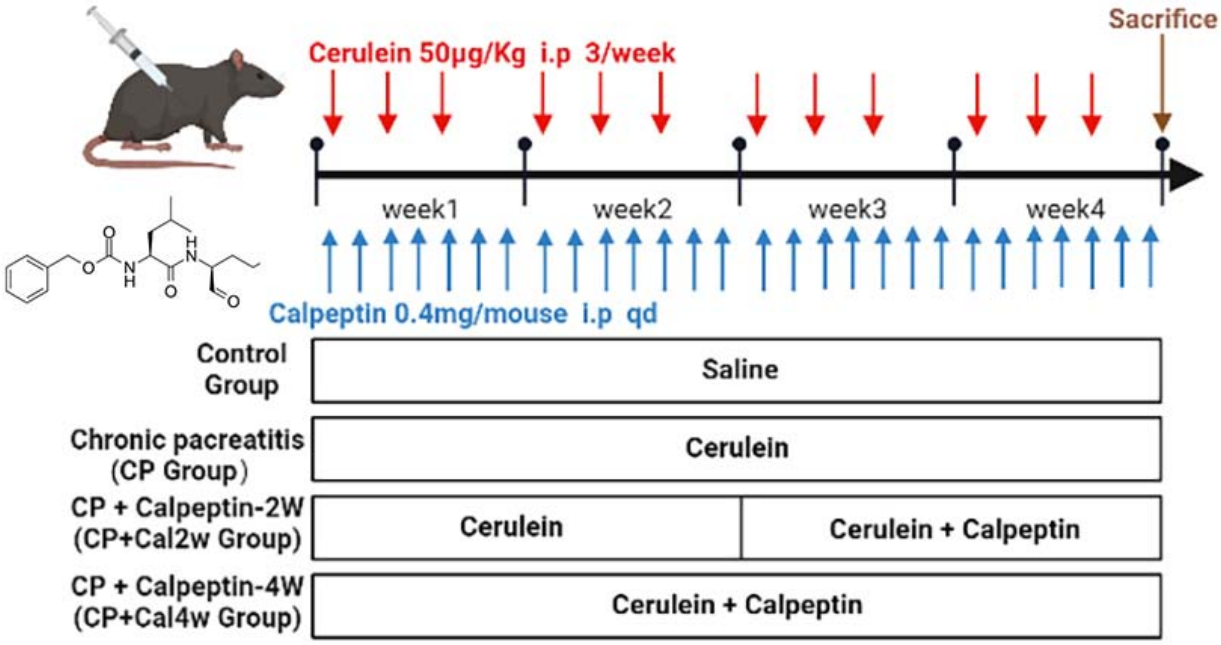


Fig. (1). Schematic chart for the experimental design. Four groups of mice (*n*=10) were analyzed. The control group received six injections of saline, while the CP group received six injections of cerulein at a dose of 50 μg/kg for three days a week. The CP+Cal2W group was given six injections of cerulein from the first day of the experimental period for a total of four weeks, and calpeptin was administered from the first day of week three until the end of the experiment, for over a total period of two weeks. The CP+Cal4W group received the same amounts of cerulein injections and calpeptin from the first day of the experimental period for a total of four weeks.

2.6. Immunohistochemical Study of Collagen-Iα1 (Col1α1), α-SMA, Calpain-1 and Calpain-2

Formalin-fixed and paraffin-embedded samples were cut into 5 μm thick slices. The tissue was then dewaxed and rehydrated with upgraded ethanol. The slide was boiled in sodium citrate solution (pH 6.0) for antigen repair in the microwave for 10 minutes. Then, the endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide solution at room temperature for 15 minutes. After rinsing with PBS, the slides were closed with bovine serum albumin (BSA) from PBS for 30 min. The sections were then incubated overnight at 4°C with alpha-SMA, Col1α1, calpain-1, and calpain-2 antibodies. Envision Detection Systems Peroxidase/DAB and Rabbit/Mouse kit (Gene Tech, Shanghai, China) were used to detect antibody binding. Next, the slices are restained with hematoxylin. Then, PBS was used as a negative control instead of a primary antibody. At the same time, the microscope (CTR 6000; Leica, Wetzlar, Germany) was used to observe the positive staining areas of α-SMA, Col1α1, calpain-1, and calpain-2 in all specimens.

2.7. Cell Culture and Calpeptin Treatment

As previously described, PSCs were isolated from male C57BL/6J mice by pancreatic tissue digestion and Nycodenz density gradient centrifugation [18, 19]. Then, freshly isolated mouse PSCs were cultured in DMEM/F12 (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). They were then incubated at 37°C and 5% CO_2. PSC culture-medium was altered and treated with different doses of calpeptin (0, 25, 50, and 100 μmol/ L) or no changes at all for four consecutive days from day 2 to day 5.

2.8. Immunofluorescence Staining

As previously described, freshly isolated mouse PSCs were cultured. On day 5, cells were harvested, washed with PBS (3x5 min), and fixed in 4% paraformaldehyde for 10 min. After washing in PBS (3x5 min), the cells were sealed with 5% BSA for 20 min and incubated with Col1α1, vimentin, and α-SMA antibodies overnight. The next day, the cells were washed in PBS (3x5 min) and then applied with the fluorescent dye Cy3 (Jackson ImmunoResearch Laboratory, USA) conjugate donkey anti-goat antibodies (1:400 dilution) and donkey anti-rabbit antibodies coupled with fluorescent dye Alexa FluorH488 (Jackson ImmunoResearch Laboratory) (1:200 dilution). For immunofluorescence detection of tissue slices, the cells were washed in PBS (3 x 5 min) and fixed in 49, 6-diamine-2-phenylindoles (DAPI) (1:1,000 dilution, Sigma-Aldrich, St. Louis), Missouri, USA. Then, confocal laser scanning microscopy (LSM710; Zeiss, Germany) and Zen 2011 software (Karl-Zeiss, Jena, Germany) were used for fluorescence analysis.

Table 1. Primer sequences used for the RT-PCR analysis.

Gene	Forward Primer	Reverse Primer
Vimentin	5'-CGGAAAGTGGAATCCTTGCAGG-3'	5'-AGCAGTGAGGTCAGGCTTGGAA-3'
Col1α1	5'-CCTCAGGGTATTGCTGGACAAC-3'	5'-TTGATCCAGAAGGACCTTGTTTG-3'
α-SMA	5'-CTGGTATTGTGCTGGACTCTG-3'	5'-GATCTTCATGAGGTAGTCGGTG-3'
Calpain-1	5'-CCTTGTTCAGCAAGTTGGCAGG-3'	5'-TCCAGGCTGAAGCCATTAGTGC-3'
Calpain-2	5'-TACCTTCCTGGTGGGTCTCATC-3'	5'-TTTGCCGAGGTGGATGTTGGTC-3'
TGF-β1	5'-TGATACGCCTGAGTGGCTGTCT-3'	5'-CACAAGAGCAGTGAGCGCTGAA-3'
Smad3	5'-AATTCTAGGCAGGTCTACTCTTGG-3'	5'-AGATGCTGTTCATACGAAAAGGAG-3'
GAPDH	5'-CATCACTGCCACCCAGAAGACTG-3'	5'-ATGCCAGTGAGCTTCCCGTTCAG-3'

2.9. Real-time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from pancreatic mouse PSC using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using the PrimeScript RT kit (Takara, Japan) according to the manufacturer's instructions. Then, according to the SYBR Premix EX Taq Manual (Takara), ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) performed RT-PCR in triplicate for each gene of interest. GAPDH was used as a single endogenous control to normalize these to genes of interest and to calculate multiple changes in gene expression using comparative CT (2−ΔΔCT) methods. Primer sequences of biomarkers were designed using software, as shown in Table **1**.

2.10. Western Blot Analysis

For western blotting, mouse pancreatic tissue was removed from storage and quickly ground in liquid nitrogen. The resulting powder or PSC is then cleaved using a nuclear and cytoplasmic protein extraction kit (Beyotime, Shanghai, China) according to the manufacturer's protocol (Pierce, CA, USA). Whole proteins obtained from pancreatic tissue or PSC were reconstructed in a cold RIPA buffer containing a mixture of benzyl methylsulfonyl fluoride (PMSF, 1 mmol/L) and protease inhibitors (1:100 dilution, Sigma-Aldrich). The homogenate was prepared by centrifugation at 4°C at 12,000 g for 15 min. Protein concentrations were then determined using the BCA method (Beyotime, Shanghai, China). Next, the same amount of protein is electrophoretic using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE Bio-Rad, CA, USA) and transferred to the membrane according to standard methods. Then, the nonspecific binding was closed in a covered container with 5% low-fat milk at room temperature for 1 hour. The membrane was then diluted at 4°C with various antibodies. The next day, goat anti-rabbit Igg-horseradish peroxidase (HRP) secondary antibody (1:2,000) and goat anti-mouse IgG-HRP antibody (1:2,000) (Santa Cruz Biotechnology, CA, USA) were applied, and the samples were left for one hour at room temperature. Finally, the membranes were cleaned and developed using the ECL assay system (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.11. Cell Proliferation

Freshly isolated mouse PSCS were inoculated into 24-well plates at a density of 2×10^5 cells per well and cultured as previously described. After 24 hours, the cells were treated as directed and incubated for four days. According to the manufacturer's instructions, proliferation was then measured using Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, St. Louis, MO, USA). Finally, the proliferating cells were counted at 200x magnification in 10 different fields in three separate experiments.

2.12. Apoptosis

Freshly isolated mouse PSCs were inoculated into 24-well plates at a density of 2×10^5 cells per well and cultured as previously described. The cells were then stained with 10 mg/ml Hoechst 33342 and examined directly using FL. After that, cells with concentrated and/or fragmented chromatin in the nucleus are considered apoptotic by looking at the cells using a microscope [20]. In three separate experiments, live cells (Hoechst negative/dark blue) and apoptotic cells (Hoechst positive/light blue) were counted in 10 different fields per well at 200x magnification.

2.13. Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance, followed by Student-Newman-Keuls (SNK) as a post hoc test. The Kruskal-Wallis test was used to assess differences in classification values, followed by the Mann-Whitney U-post hoc test. When $p<0.05$, the data were considered statistically significant.

Table 2. Calpeptin reduces the severity of experimental cerulein-induced CP.

-	Control	CP	CP+Calpeptin2W	CP+Calpeptin4W
Glandular atrophy	0 ± 0	2.89 ± 0.45*	1.57 ± 0.35$^{\#}$	1.38 ± 0.24$^{\#}$
Fibrosis	0 ± 0	2.54 ± 0.35*	1.42 ± 0.24$^{\#}$	1.20 ± 0.19$^{\#}$
Inflammation	0 ± 0	2.68 ± 0.26*	1.64 ± 0.31$^{\#}$	1.38 ± 0.22$^{\#}$
Damage index	0 ± 0	7.09 ± 0.45*	4.63 ± 0.35$^{\#}$	3.98 ± 0.23$^{\#}$

Note: The data was presented as the mean ± standard deviation (SD) of three independent experiments. *$p<0.05$, compared to the control and RA groups; $^{\#}p<0.05$, compared to the cerulein group.

3. RESULTS

3.1. Calpeptin Ameliorates Cerulein-induced Chronic Pancreatitis and Attenuates Fibrosis in Pancreatic Tissues

After the induction by cerulein for four weeks, compared to the control group, the CP and calpeptin groups presented with obvious histological features at the time of sacrifice (week 4), including structural abnormalities, glandular atrophy, pseudotubular complexes, fibrosis, and inflammatory cell infiltration. Indeed, the CP+Cal2W and CP+Cal4W groups presented less damage based on the histological scores in the pancreas (Table **2** and Fig. **2A**).

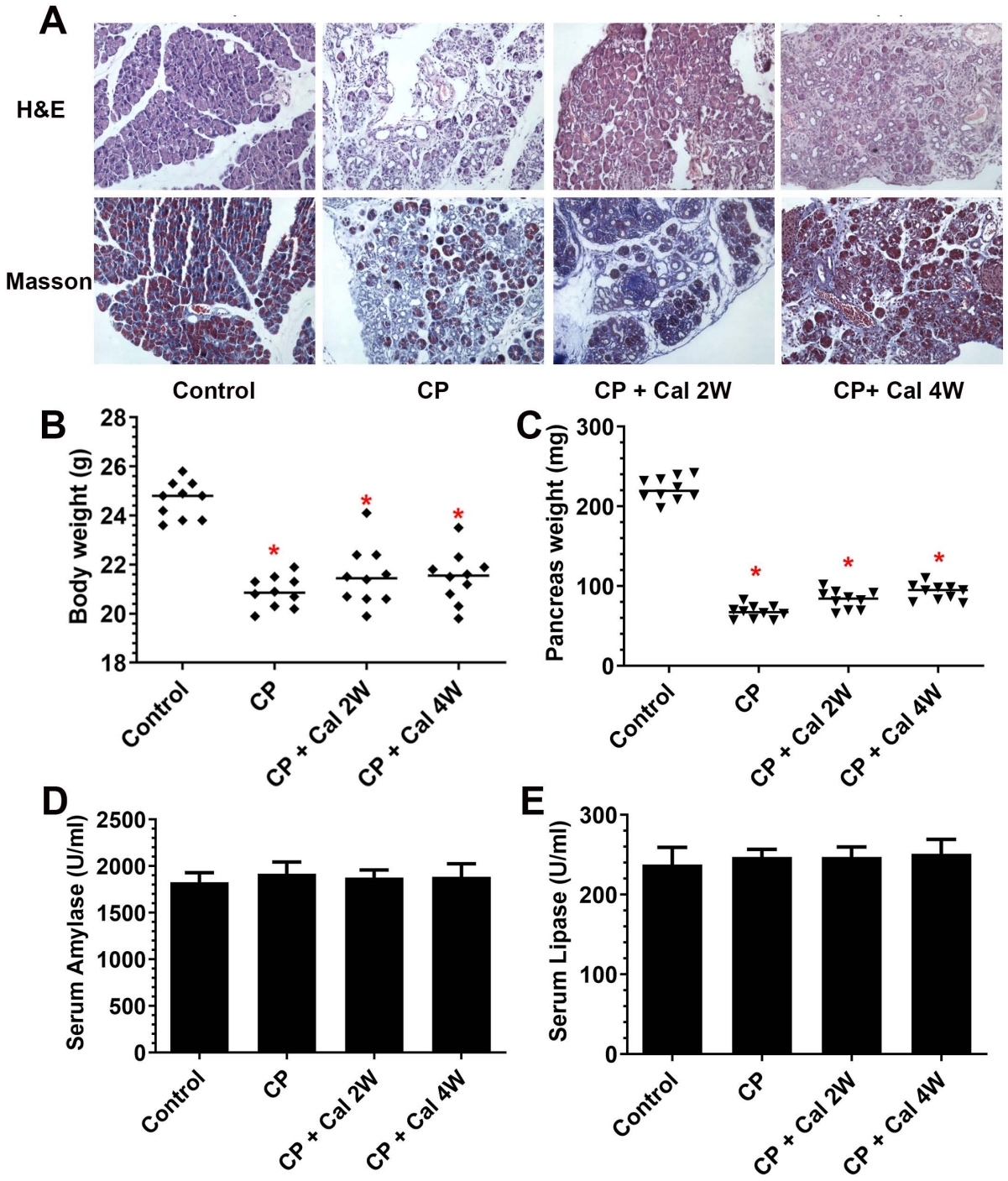


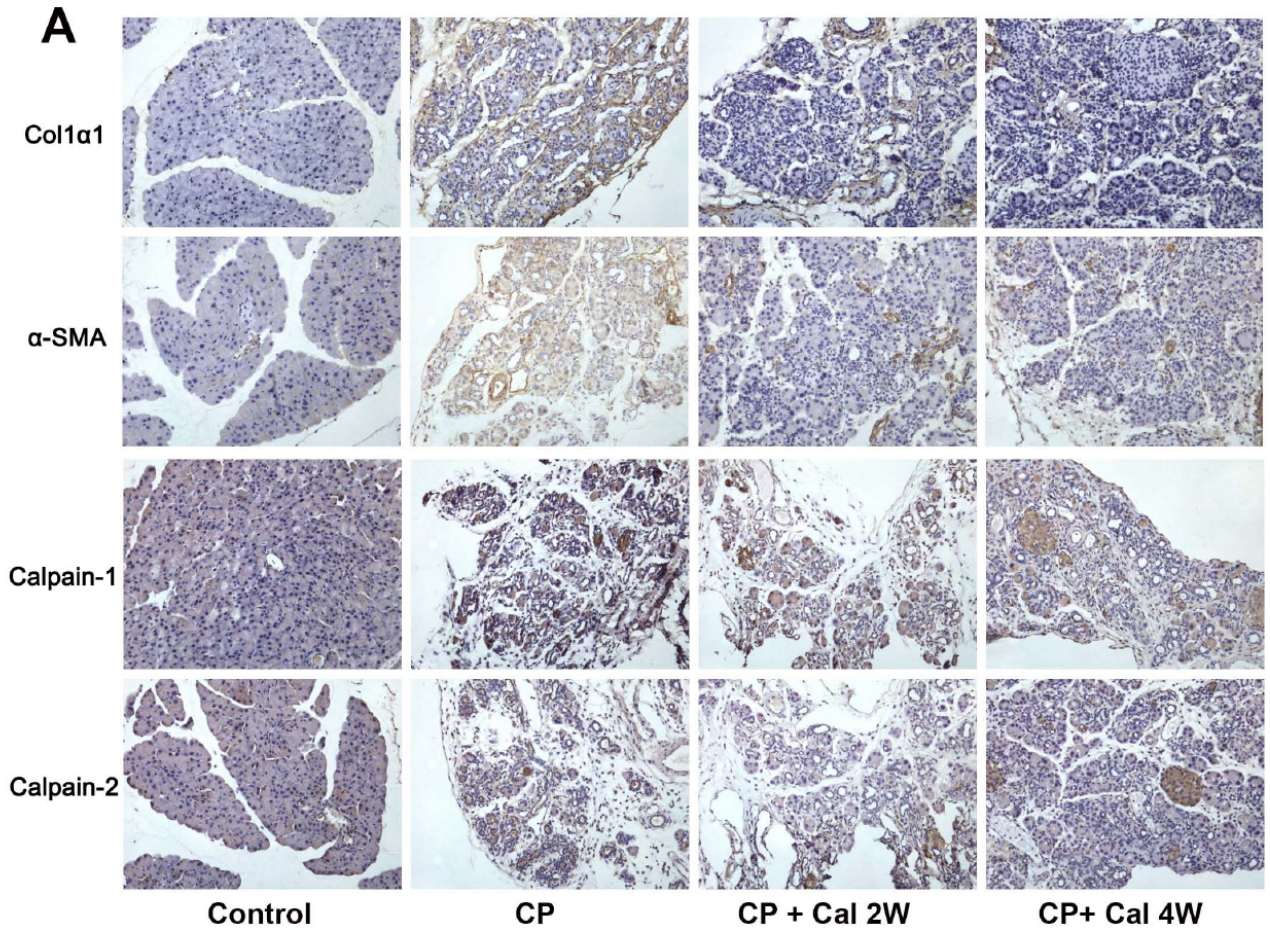
Fig. (2). Assessment of chronic pancreatitis in mice. (**A**) Pancreatic tissues in all groups were evaluated *via* H&E staining and Masson staining (magnification: ×100). (**B** and **C**) The assessment of the body weight and pancreas weight among the four groups of mice in the 4-week experimental period revealed no changes among the control, CP and calpeptin groups. (**D** and **E**) The activities of serum amylase and lipase were analyzed. No obvious changes were evident among the groups.

The pancreatic sections stained with Masson were analyzed to assess the level of fibrosis. The pancreatic collagen content significantly increased after the induction of mice CP, and this improved after the administration of calpeptin (Fig. **2A**). The positive area of Masson staining was higher in the CP group when compared to the control group. In comparison, the positive area of Masson staining was smaller in the CP+Cal2W and CP+Cal4W groups when compared to the CP group (Fig. **2A**).

After the long-term observation, the body weight and pancreas weight of mice with cerulein-induced chronic pancreatitis significantly decreased, while mice in the calpeptin-treated group presented with no significant increase in body weight when compared to mice in the CP group (Fig. **2B**). Furthermore, the 2- weeks and 4-weeks treatment in the calpeptin group had no significant effect on the decrease in pancreas weight induced by cerulein at week four (Fig. **2C**). Moreover, there were no differences in changes in serum amylase and lipase levels among the groups (Fig. **2D** and **2E**).

3.2. Calpeptin Reduces Fibrosis in Pancreatic Tissues and Inhibits Calpain-1 and Calpain-2 Expression in Chronic Pancreatitis

Consistent with Mason's staining, the extent of the pancreatic fibrosis was also indicated by the immunohistochemistry for Col1α1 and α-SMA in pancreatic tissues. Compared to the control group, this was significantly higher in the CP group, while the collagen expression decreased in pancreatic tissues in the calpeptin group (Fig. **3A**). The immunohistochemistry for Col1α1 and α-SMA was performed to quantify the number of activated stellate cells. Compared to the control group, the expression significantly increased in the CP group, while this significantly decreased in the calpeptin groups (Fig. **3A**).



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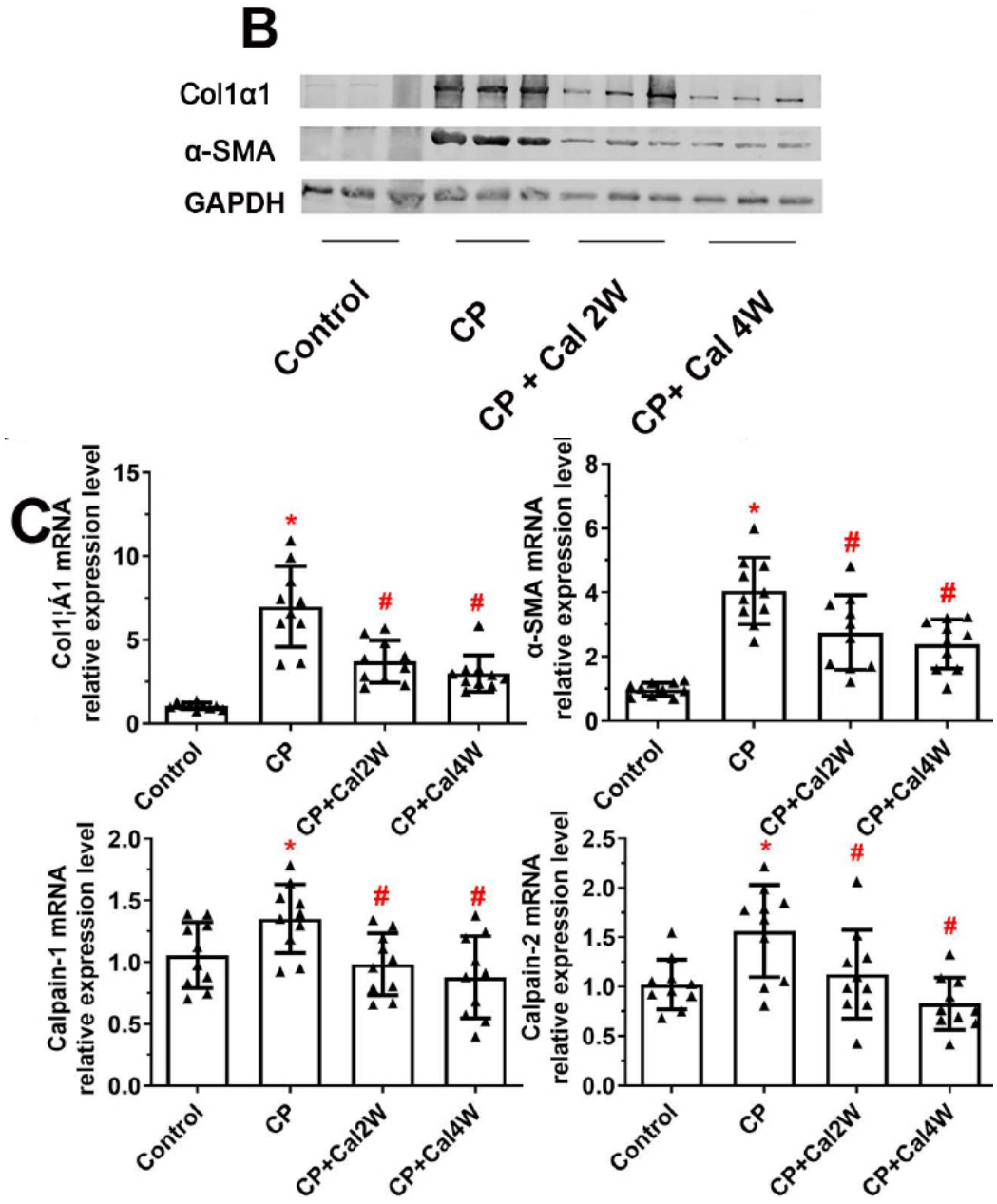


Fig. (3). Calpeptin attenuates pancreatic fibrosis in cerulein-induced CP mice. (**A**) The immunochemical staining of pancreatic tissue sections for Col1α1, α-SMA, calpain-1 and calpain-2 (magnification: ×100). (**B**) The western blot analysis of the Col1α1 and α-SMA protein expression in the four groups. GAPDH was used as the internal reference. (**C**) RT-PCR analysis of the mRNA levels of Col1α1, α-SMA, calpain-1 and calpain-2. GAPDH was used as the housekeeping control. The data were presented as the mean ± standard deviation (SD) of three independent experiments. *p<0.05, compared to the control group, and $^{\#}p$<0.05, compared to the CP group.

Western blotting showed that Col1α1 and α-SMA were upregulated in the CP group compared with the control group. However, this upregulation was significantly inhibited in both CP+Cal2W and CP+Cal4W groups (Fig. **3B**). Consistently, RT-PCR results showed that mRNA expression levels of Col1α1 and α-SMA were significantly increased compared with the control group and CP group. However, this upregulation was significantly inhibited in both CP+Cal2W and CP+Cal4W groups (Fig. **3C**).

In order to determine whether calpeptin (the calpain gene inhibitor) affects the expression of calpain-1 and calpain-2, the translocation of calpain-1 and calpain-2 was examined by immunohistochemical staining and RT-PCR. The immunohistochemical staining revealed that the expression of calpain-1 and calpain-2 significantly increased in the CP group compared to the control group. However, this was significantly suppressed in the calpeptin group (Fig. **3A**). Correspondingly, in the RT-PCR experiments, the mRNA expression levels of calpain-1 and calpain-2 were upregulated in the CP group but were significantly suppressed in the calpeptin group (Fig. **3C**).

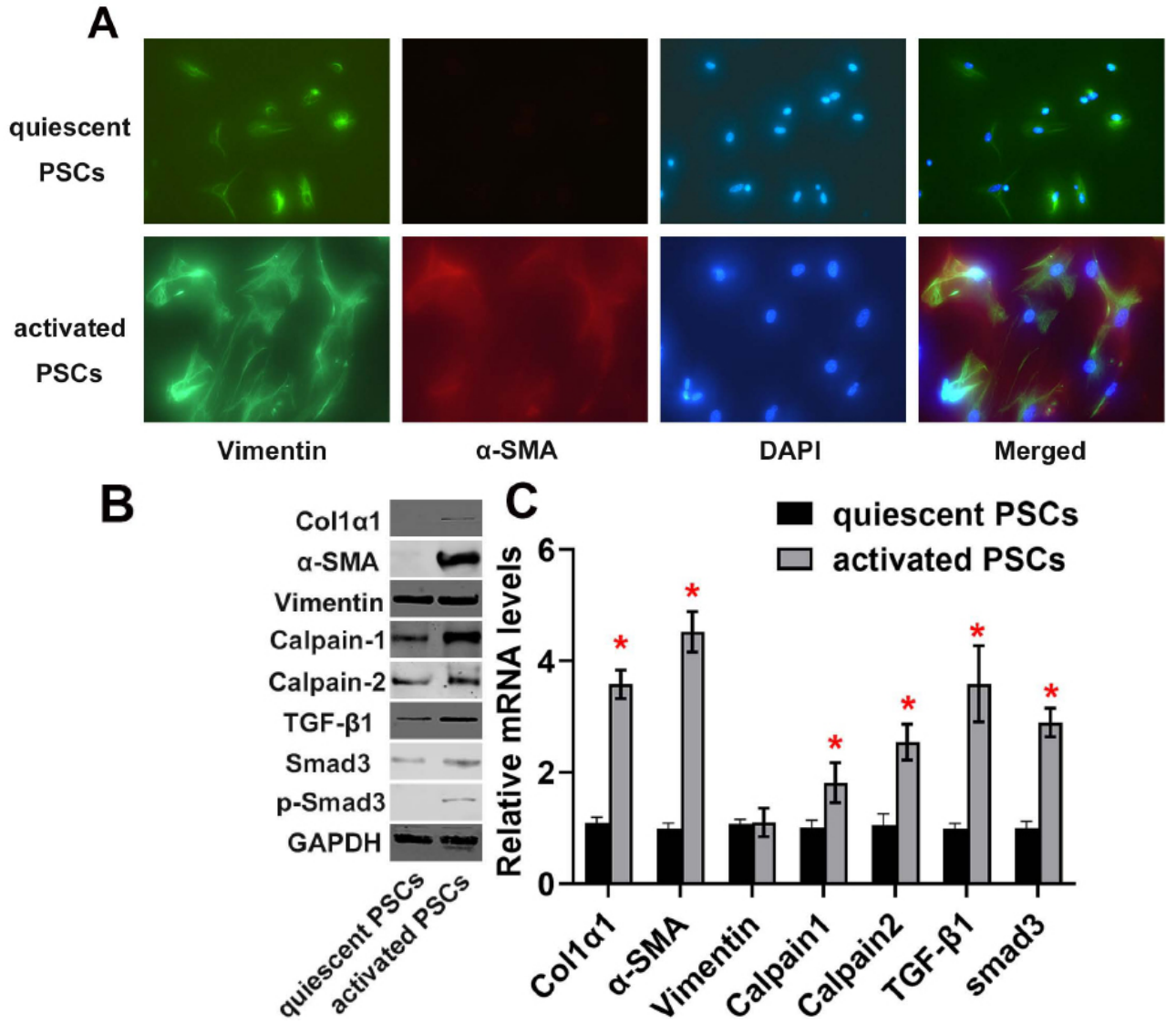


Fig. (4). The expression of calpain-1 and calpain-2 increased during PSCs activation *in vitro*, and the expression of TGF-β1/smad3 also increased. (**A**) The immunofluoresence analysis of vimentin and a-SMA in quiescent cells and activated pancreatic stellate cells (PSCs) is shown (magnification: ×400). (**B**) The protein levels of Col1α1, α-SMA, vimentin, calpain-1, calpain-2, TGF-β1, smad3 and phosphorylated-smad3 in quiescent and activated PSCs were detected by western blot. GAPDH was used as the internal reference. (**C**) The real-time polymerase chain reaction (RT-PCR) for Col1α1, α-SMA, vimentin, calpain-1, calpain-2, TGF-β1 and smad3 demonstrated differences between quiescent and activated PSCs. The statistically significant differences between quiescent and activated PSCs were indicated as *p<0.05.

3.3. The Expression of Calpain-1 and Calpain-2 Increased During PSC Activation *In Vitro*, and the Expression of TGF-β1/smad3 also Increased

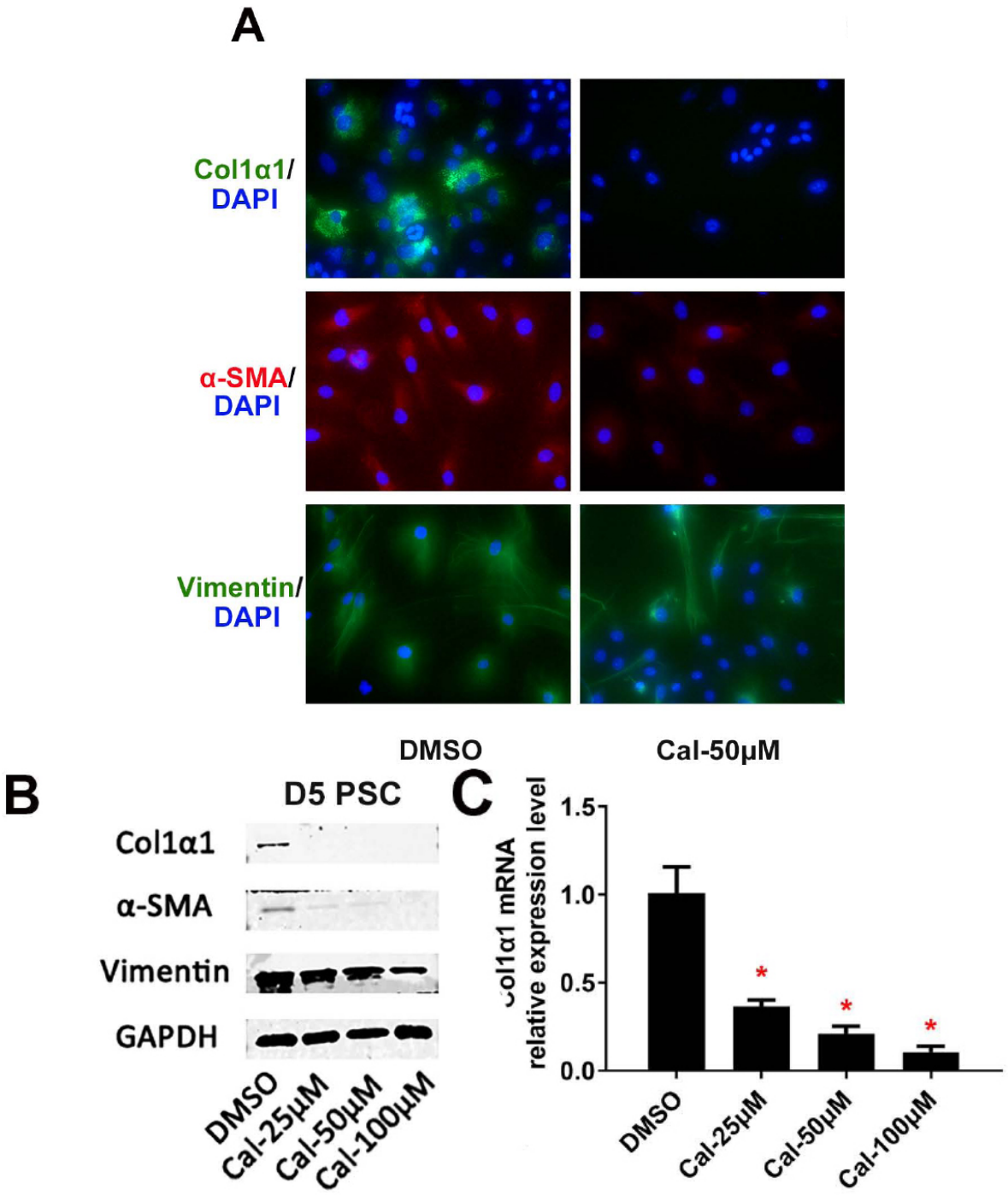
The morphological changes of PSCs during activation were observed by culturing PSCs *in vitro*. From the immunofluorescence of cells, it was found that PSCs changed from quiescent cells to activated cells, and the morphology changed to fibroblasts.

The expression of α-SMA significantly increased, while the expression of the PSC marker, vimentin, did not change substantially (Fig. **4A**). Meanwhile, the western blot and RT-PCR confirmed the increase in α-SMA expression. It was further revealed that the protein and mRNA levels of Col1α1, calpain-1 and calpain-2 increased during PSC activation. On the contrary, the expression of TGF-β1 and smad3 significantly increased (Fig. **4B** and **C**).

3.4. Calpeptin Inhibited PSC Activation and Proliferation and Promoted PSC Apoptosis *In Vitro*

As previously mentioned, Day 1 PSCs were incubated with or without calpeptin for four days at varying doses (0, 25, 50, 100 μmol/L). To compare day 1 PSCs, day 5 PSCs were collected and, as previously described, used for immunofluorescence staining, western blotting, and RT-PCR analysis. Immunofluorescence staining showed that calpeptin inhibited the expression of α-SMA and Col1α1 during the activation of PSCs (Fig. **5A**). In addition, calpeptin induced significant downregulation of α-SMA and Col1α1 at the

protein and mRNA levels during PSCs activation but did not induce vimentin expression (Fig. **5B** and **C**). Taken together, these findings suggest that calpeptin can significantly inhibit PSC activation *in vitro*.



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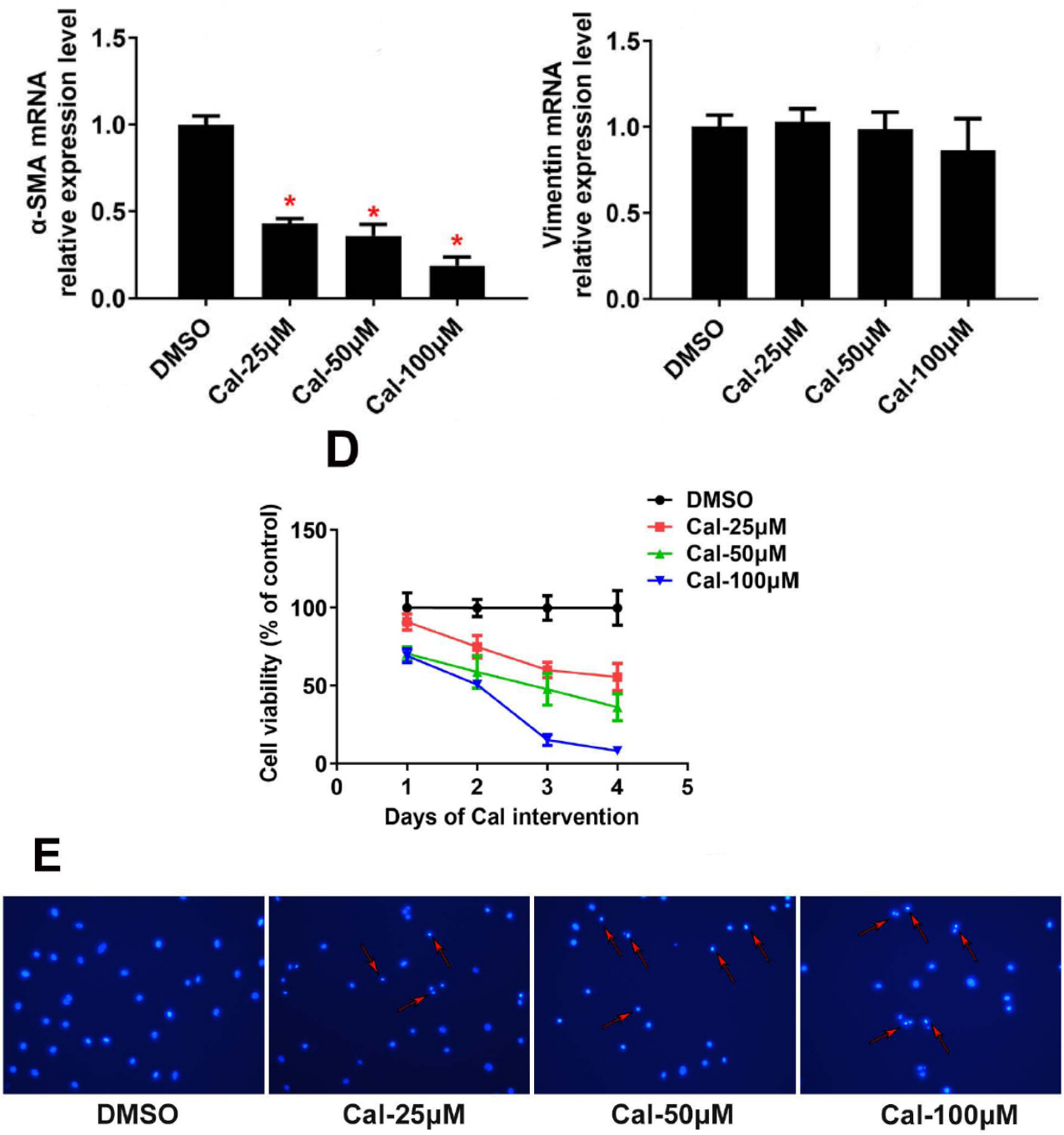

Fig. (5). Calpeptin inhibits pancreatic stellate cells (PSCs) activation and proliferation, and promotes the apoptosis of PSCs *in vitro*. The immunofluoresence analysis of Col1α1, a-SMA and vimentin in PSCs treated with calpeptin is shown (magnification: ×400). (**B**) The protein levels of Col1α1, α-SMA and vimentin in PSCs treated with calpeptin were detected by western blot. GAPDH was used as the internal reference. (**C**) The real-time polymerase chain reaction (RT-PCR) for Col1α1, α-SMA and vimentin in PSCs treated with calpeptin is shown. Statistically significant differences between quiescent cells and activated PSCs were indicated as *p<0.05. (**D**) Day 1 PSCs were cultured with different doses of calpeptin (0, 25, 50 and 100 μmol/L) for four days, and the cell viability was detected using Cell Counting Kit-8 (CCK-8). (**E**) The Hoechst 33342 staining is shown (magnification: ×400). The data was presented as the mean ± standard deviation (SD) of three independent experiments. *p<0.05, compared to the control group.

PSCs were harvested (days 2-5), and the cell proliferation was analyzed using the sensitive colorimetric method, CCK-8. The addition of calpeptin inhibited PSC proliferation. Interestingly, a dose-dependent effect was also observed. Calpeptin-induced inhibition became significant at a dose of 50 μmol/L (Fig. **5D**). Furthermore, the effect of calpeptin on the apoptosis of PSCs was investigated by Hoechst 33342 staining. The results collectively demonstrated that calpeptin can significantly promote the apoptosis of PSCs *in vitro* (Fig. **5E**).

3.5. Calpeptin Inhibits Calpain-1 and Calpain-2 Expression and Modulates the TGF-β1/smad3 Signaling Pathway in PSCs Activation

In order to determine whether the calpain gene inhibitor, calpeptin, affects the TGF-β1 and smad3 expression, the expression of calpain-1, calpain-2, TGF-β1 and smad3 were detected by western blot and RT-PCR. In the Western blot assay, compared to the control group, the protein expression of calpain-1, calpain-2, TGF-β1 and smad3 was significantly inhibited in the calpeptin group, and the expression of phosphorylated Smad3 was also reduced by calpeptin (Fig. **6A**). Correspondingly, the RT-PCR results revealed that the mRNA expression levels of these genes all decreased in different degrees in the calpeptin group (Fig. **6B**).

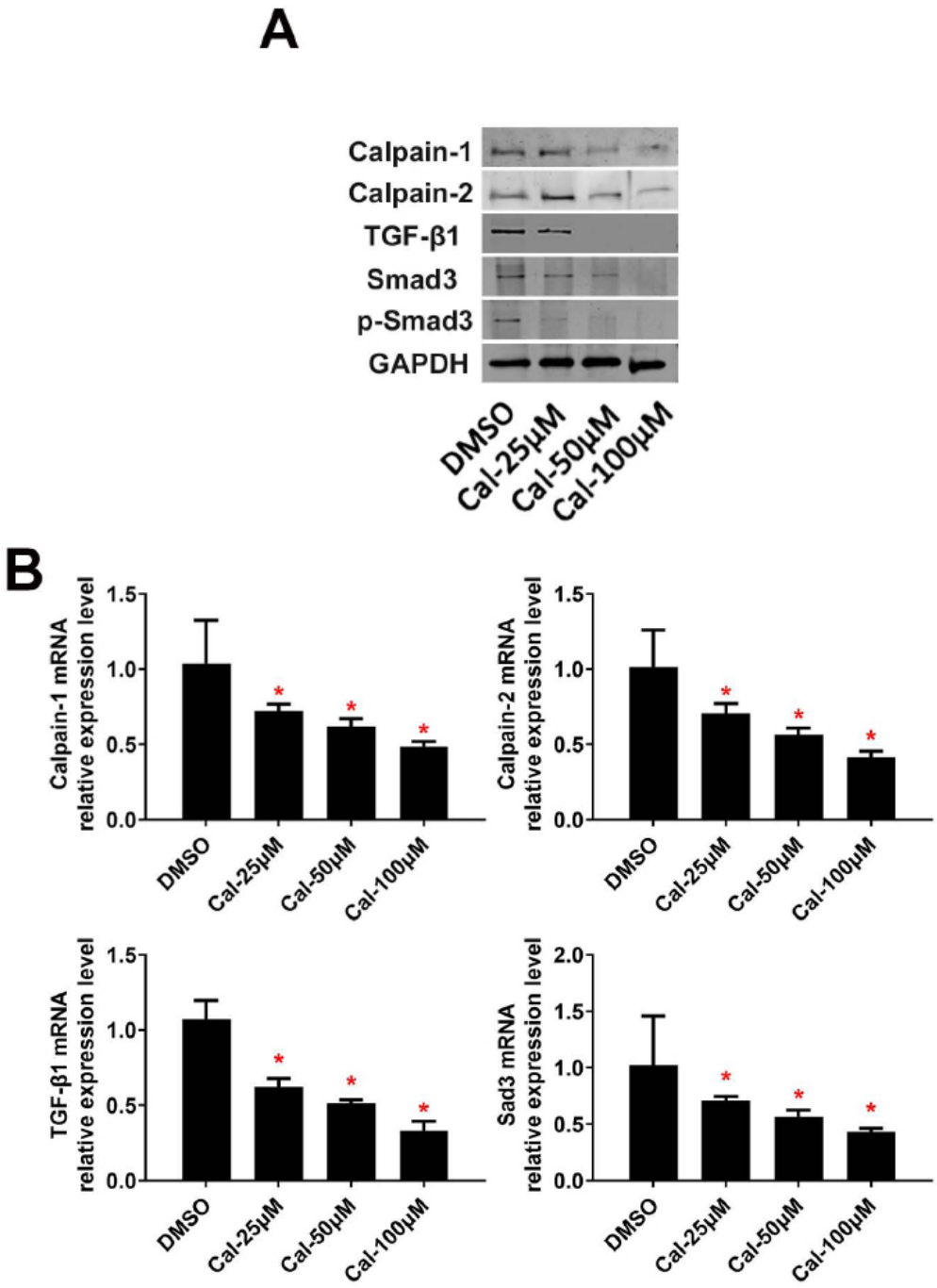

Fig. (6). Calpeptin inhibits the expression of calpain-1 and calpain-2, and modulates the TGF-β1/smad3 signaling pathway. The protein levels of calpain-1, calpain-2, TGF-β1, smad3 and phosphorylated-smad3 in pancreatic stellate cells (PSCs) treated with calpeptin were detected by western blot. GAPDH was used as the internal reference. (**B**) The real-time polymerase chain reaction (RT-PCR) for calpain-1, calpain-2, TGF-β1 and smad3 in PSCs treated with calpeptin is shown. The statistically significant differences between quiescent cells and activated PSCs were indicated as *$p<0.05$.

4. DISCUSSION

Pancreatic fibrosis is a prominent feature of CP, causing persistent and permanent damage to the pancreas. This study suggests that PSCs are the main source of ECM deposition during pancreatic injury, and the continuous activation of PSCs plays a crucial role in the progression of pancreatic fibrosis. One of the main features of PSC activation is a gradual reduction in the size and number of fat droplets containing retinoic acid, along with a transition of PSCs to myofibroblast [21]. Recent studies have uncovered some new mechanisms of pancreatic fibrosis. The study of Jiang identified a protective role of the pancreatic clock against pancreatic fibrosis and exocrine dysfunction [22]. VLDLR-enhanced lipoprotein metabolism in PSCs promotes pancreatic fibrosis [23]. High pressure within the pancreas stimulates Piezo1 channel opening, leading to stellate cell activation and pressure-induced chronic pancreatitis and fibrosis [24].

According to related studies, calpeptin is a cell-penetrating calpain inhibitor and plays a role in certain organ fibrosis. Furthermore, calpeptin can prevent moramycin-induced pulmonary fibrosis in mice [5], and this mechanism may be through the switching in the development of epithelial-mesenchymal transition6. Recent studies have shown that calpain could promote idiopathic pulmonary fibrosis by migrating pleural mesothelial cells into the lung parenchyma [25]. Another study has revealed that calpeptin can induce the apoptosis of hepatic stellate cells by inhibiting the calpain/caspase-12 pathway [26]. In the later stages of skin healing, calpain inhibition has been shown to be beneficial by reducing collagen production and wound fibrosis, thereby inhibiting cell migration and invasion [27]. At the same time, calpeptin has been found to inhibit the progression of certain tumors. Calpeptin can prevent malignant pleural mesothelioma cell proliferation through the angiopoietin 1/TIE2 system [28]. Furthermore, calpeptin could inhibit pancreatic cancer by disrupting cancer-stroma interactions in a mouse xenograft model, and the mechanism was by inhibiting tumor-related pancreatic stellate cells' (PSCs) biological functions, including proliferation, migration and invasion *in vitro* [29].

However, the specific role of calpeptin in pancreatic stellate cells (PSCs) and pancreatic fibrosis, especially CP, has not been determined. Our study used an *in vivo* cerulein-induced CP mouse model and the *in vitro* activation process of mouse PSCs and observed the effect of calpeptin. The results showed that calpeptin not only improved the progression of pancreatic fibrosis in experimental CP but also inhibited the activation process of PSCs *in vitro*. In addition, it was found that calpeptin induced the PSCs activation by inhibiting the calpain-1 and calpain-2 expression and inhibiting TGFβ1-Smad3 signaling pathways.

The CP mouse model was established successfully, and it was found that inhibiting calpain could improve the progression of CP and the degree of pancreatic fibrosis. CP mouse models involving repeated administration of azurin (an analog of ecocystokinin 8 exocrine) resulted in increased acinar damage, ECM deposition, and fibrotic lesions [30]. In the present study, the H&E, Masson, Col1α1, and α-SMA staining experiments revealed that the pancreatic damage and fibrosis were reduced after the administration of calpeptin *in vivo,* indicating that the progression of pancreatic fibrosis induced by cerulein was attenuated in CP mice. In addition, calpain-1 and calpain-2 were identified as the major isoforms of conventional calpain, which calpeptin can effectively inhibit. Therefore, it was confirmed that calpeptin protects the progression of CP and pancreatic fibrosis.

Our study was the first to reveal that calpain-1 and calpain-2 are activated during the activation of PSCs *in vitro*. In the process of PSC activation, in addition to the upregulation of fibrosis-related genes Col1α1 and α-SMA, the TGF-β1/smd3 genes were also upregulated. These results are consistent with the known pro-fibrotic effects of calpain in different organs in a tissue-specific manner. For example, the crosstalk between calpain activation and TGF-β1 enhances the collagen-I synthesis in pulmonary fibrosis [7]. Furthermore, the calpain small subunit plays an important role in mechanical force generation and mediates the mechanosensing during fibroblast migration [31]. However, in mice knockout calpain-9, the loss of calpain-9 was shown to play a role in bleomycin-induced lung fibrosis, carbon tetrachloride-induced liver fibrosis, and angiotensin II-induced cardiac fibrosis and dysfunction [32].

Our study also revealed that calpeptin could inhibit the TGFβ-1/Smad3 signaling pathway in the process of PSCs activation. The TGFβ-1 function is critical in fibrosis [33]. Given that TGFβ-1 acts as the “master switch” for PSC activation during pancreatic fibrosis, it was determined whether calpeptin inhibits the TGFβ1 expression. It was found that calpeptin inhibits the expression of calpain-1 and calpain-2 and inhibits the level of TGF-β1 and phosphorylation of Smad3, suggesting that calpain may be the upstream of TGF-β1. It was reported that the overexpression of calpain-1 induces TGFβ1-Smad signaling in age-related central arterial wall stiffness [34]. Consistently, it has been revealed that calpain could cleave and activate intracellular latent TGF-β1 and initiate the TGF-β1 pathway [35].

CONCLUSION

In conclusion, calpeptin can improve the degree of pancreatic fibrosis in mouse CP models, inhibit its activation or *in vitro* activation of PSCs, and inhibit the expression of Col1α1, α-SMA, and other fibrosis markers in PSCs. The mechanism may be correlated to the inhibition of calpain-1 and calpain-2 to activate the TGFβ1/smad3 signaling pathway. Therefore, aiming to improve the function of the calpain inhibitor, calpeptin, in the CP model may be a novel clinical treatment approach for CP, which can be further developed.

LIST OF ABBREVIATIONS

CP	=	Chronic Pancreatitis
PSCs	=	Pancreatic Stellate Cells
ECM	=	Extracellular Matrix
α-SMA	=	α-smooth muscle actin

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal-related procedures were approved by the

Animal Care and Use Committee of Shanghai Shanghai General Hospital, Shanghai Jiaotong University School of Medicine (Approval no. 2019AW059).

HUMAN AND ANIMAL RIGHTS

No humans were involved in this study. All animal procedures were performed in accordance with the eighth edition of the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (The National Academies Press, Washington, DC, USA).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

This work was sponsored by the National Natural Science Foundation of China (81500487, 82100683, 82170653, and 82270673) and the Shanghai Sailing Program (20YF1439600)

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was sponsored by the National Natural Science Foundation of China and the Shanghai Sailing Program.

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