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Catechin hydrate ameliorates cerulein-induced chronic pancreatitis via the inactivation of TGF-β/Smad2 signaling

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Abstract. Chronic pancreatitis (CP) is a pancreatic inflammatory disease associated with histological changes, including fibrosis, acinar cell loss and immune cell infiltration, and leads to damage of the pancreas, which results in pain, weight loss and loss of pancreas function. Catechin or catechin hydrate (CH) has antioxidant, anticancer and immune-regulatory effects. However, unlike other catechins, the antifibrotic effects of (+)-CH have not been widely studied in many diseases, including CP. Therefore, the anti-fibrotic effects of (+)-CH against CP were evaluated in the present study. To assess the prophylactic effects of CH, (+)-CH (1, 5 or 10 mg/kg) or ethanol was administered 1 h before first cerulein (50 μg/kg) injection. To assess the therapeutic effects, (+)-CH (5 mg/kg) or ethanol was administered after cerulein injection for one or two weeks. In both methods, cerulein was injected intraperitoneally into mice once every hour, six times a day, four times a week, for a total of three weeks, to induce CP. The data showed that (+)-CH markedly inhibited glandular destruction and inflammation during CP. Moreover, (+)-CH prevented pancreatic stellate cell (PSC) activation and the production of extracellular matrix components, such as fibronectin 1 and collagens, which suggested that it may act as a novel therapeutic agent. Furthermore, the mechanism and effectiveness of (+)-CH on pancreatic fibrosis were investigated in isolated PSCs. (+)-CH suppressed the activation of Smad2 and fibrosis factors that act through transforming growth factor-β (TGF-β) or platelet-derived growth factor. These findings suggest that (+)-CH exhibits antifibrotic effects in cerulein-induced CP by inactivating TGF-β/Smad2 signaling.

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Key words: chronic pancreatitis, (+)-catechin hydrate, pancreatic fibrosis, cerulein, pancreatic stellate cells

Introduction

Chronic pancreatitis (CP), considered as a main cause of pancreatic cancer responsible for high morbidity, is associated with an increased incidence and prevalence worldwide (1,2). CP is a pancreatic inflammatory disease characterized by histological changes, including fibrosis, acinar cell loss and immune cell infiltration and eventually leads to damage of the pancreas, resulting in numerous downstream effects (3). CP not only reduces the quality of life of patients due to pain, weight loss and malnutrition but is also a putative risk factor for pancreatic cancer and diabetes and their consequent complications (4-9). Recent research has suggested that in South Korea, the incidence of cancer and the resultant mortality rate are 1.2 times higher in patients with CP than those in the individuals without CP (10). Frequent alcohol consumption is known to be the main cause of CP, accounting for ~80% of cases of CP development. However, the non-alcohol-dependent incidence has also been increasing recently (2,11,12). Therefore, it is imperative to develop effective CP treatments beyond lifestyle improvements.

Plants and their active ingredients which are found in food and tea, are consumed daily and have been attracting attention as valuable targets in drug development because they reportedly, prevent diseases and improve treatment effects beyond simple nutrition (13). Tea, in particular, is the most consumed beverage in the world, with the exception of water, and has beneficial effects, including anti-fibrotic, anti-inflammatory and anti-cancer effects, mainly caused by polyphenols (14-17). Catechins, which are polyphenolic flavonoids, are abundant in commonly consumed foods and herbs such as apples and tea (18). Among them, (-)-epigallocatechin-3-gallate (EGCG), a major catechin in green tea, has been reported to alleviate pancreas-related diseases, including fibrosis, inflammation and cancer (18-21). Initially, low-dose intake of catechins was considered safe, but recent studies of hepatotoxicity in catechins have raised safety concerns related to their consumption (22,23).

Catechin and catechin hydrate (CH) are natural flavon-3-ol phytochemicals and are relatively less toxic than

other flavonoids including EGCG (24-26). CH is found in *Tamarindus indica* fruit pulp, the fruit peel of *Areca catechu* Linné and in green tea (24,27,28). Beneficial effects of catechin on various diseases have been previously reported. CH has protective efficacy against benzo(a)pyrene induced lung injury by regulating apoptosis, inflammation and oxidative stress (29). It has also demonstrated anticancer effects through inhibition of the proliferation of MCF-7 cells and inducing P53 and caspase-medicated apoptosis of human breast cancer cells (26). In addition to antioxidant and apoptosis activities, the efficacy of (+)-catechin on hepatic fibrosis has been reported. Bragança de Moraes *et al* (30) reported that it reversed activated hepatic stellate cells (HSCs) to the quiescent state by regulating intracellular lipid droplets and HSC quiescent markers.

Previous studies have primarily focused on the medicinal effects of green tea extract, which were limited to assessing the effects of EGCG. Therefore, despite the aforementioned results, studies on other catechins are still lacking. In our previous study, it was demonstrated that the water extract of fruit peel of *Areca catechu* Linné contained catechin which showed remarkable inhibitory effects on pancreatic fibrosis (27). Despite this, little research has been reported regarding the effect of CH on pancreatic fibrosis. Therefore, the present study assessed the potentially beneficial effects of (+)-CH, another promising single compound, on CP.

Materials and methods

Preparations of (+)-CH. (+)-CH was purchased from MilliporeSigma (cat. no. 1251G; purity ≥98%). The powder was dissolved in ethanol (cat no. 1.00983.1011; Merck Life Science UK, Ltd.) at a concentration of 200 mg/ml to prepare the stock solution and was further diluted in saline or Dulbecco's Modified Eagle Medium (DMEM; cat. no. 11995-065; Thermo Fisher Scientific, Inc.) to obtain the desired concentrations.

Animals. All experiments were performed with the approval of The Animal Care Committee of Wonkwang University (approval no. WKU22-43; Iksan, Republic of Korea). A total of 196 C57BL/6 mice (6-8 weeks old, female, weighing 15-20 g) were purchased from Samtako Biokorea Co., Ltd. Mice were housed in standard shoebox cages in a climate-controlled room maintained at an ambient temperature of 23±2˚C and a 12 h light-dark cycle for 7 days. They were fed standard laboratory chow, offered water *ad libitum* and were arbitrarily assigned to the control and experimental groups. Isoflurane (induction, 4.5%; maintenance, 1.5%) in 95% O_2 and 5% CO_2 was used for anesthesia. CO_2 inhalation was used for euthanasia with a flow rate which displaced 50% of the cage vol/min and cervical dislocation was also performed to ensure death following CO_2 asphyxiation.

Experimental design. Mice were intraperitoneally injected with 50 μg/kg cerulein (cat. no. H-3220; Bachem AG) six times/day at 1 h intervals, four times/week, for 3 weeks. In the prophylactic treatment groups, mice were intraperitoneally injected with (+)-CH (1, 5 or 10 mg/kg) or ethanol (control group) 1 h before the first daily injection four times/week (Fig. 1A). In the curative group, (+)-CH (5 mg/kg) was administered for 2 weeks or 1 week after the first cerulein injection. After 3 weeks, the mice were sacrificed using CO_2 asphyxiation and the pancreas was collected and stored at -80˚C for further analysis.

Biochemical analysis. (+)-CH (1, 10, 20 or 50 mg/kg) or ethanol (control group) were intraperitoneally administered to mice (n=3/group). At 24 h following administration, the serum was collected by cardiac puncture and separated by centrifugation at 2,339 x g for 5 min at 4˚C. Fresh serum was analyzed using biochemical analyzer (cat. no. NX700i; FUJIFILM Wako Pure Chemical Corporation) according to the manufacturer's instructions. The levels of the following molecules were examined: Alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN) and creatinine (CREA).

Tissue processing. After obtaining fresh pancreas, tissues were washed with cold phosphate buffered saline (PBS; cat. no. CNP010-1000; Isell Co., Ltd.) and the specimens were fixed in 10% neutral-buffered-formalin solution (cat. no. HT501128; MilliporeSigma) for 24 h at room temperature. Tissues were then dehydrated using an ascending alcohol series. Subsequently, ethanol was cleared using xylene. The tissues were infiltrated with paraffin wax and embedded and sectioned into 4 μm thick sections. Formalin-fixed paraffin-embedded (FFPE) samples were deparaffinized by placing slides in xylene twice for 10 min each and rehydrated in 100% ethanol twice for 5 min each, 95% ethanol for 2 min, 70% ethanol for 2 min at room temperature. The samples were then stained using hematoxylin for 8 min and eosin for 2 min at room temperature. The experiment was performed independently, three times. The sections were imaged using NIS-Elements Viewer 4.2 (Nikon instruments). The samples were scored on a scale from 0 to 3 based on the presence of glandular atrophy and inflammation (0, normal, no glandular atrophy and inflammation; 1=mild, found in less than 25% of the pancreas. 2=moderate, found in less than 25 to 75% of the pancreas. 3=found in more than 75 % of the pancreas).

Immunohistochemical (IHC) staining. Prior to IHC staining, FFPE pancreatic tissues were deparaffinized and rehydrated. The slide was placed in 10-fold diluted citrate buffer Antigen retriever (pH 6.0, cat. no. C9999; MilliporeSigma) and heated for 20 min at 100˚C. The slides were blocked with 5% normal goat serum (Jackson ImmunoResearch Laboratories, Inc.) at RT for 1 h and incubated with a primary antibodies against α-smooth muscle actin (α-SMA; 1:100; cat. no. sc-32251; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. Following incubation with HRP-conjugated goat anti-rabbit secondary antibody (1:1,000; cat. no. SA002-500; GenDEPOT, LLC) for 1 h at RT, the slides were covered with diluted 3,3'-diaminobenzidine (DAB) solution; Dako Liquid DAB$^+$ Substrate Chromogen System (1:50; cat. no. K3467; Dako; Agilent Technologies, Inc.) and incubated for 3 min. Hematoxylin was used as the counterstain for 3 min at RT. The experiment was performed independently, three times. Samples were imaged using a light microscope Nikon Ti2-U (Nikon instruments) and the relative intensity was quantified using the microscopy software NIS-Elements Viewer 4.2 (Nikon instruments).

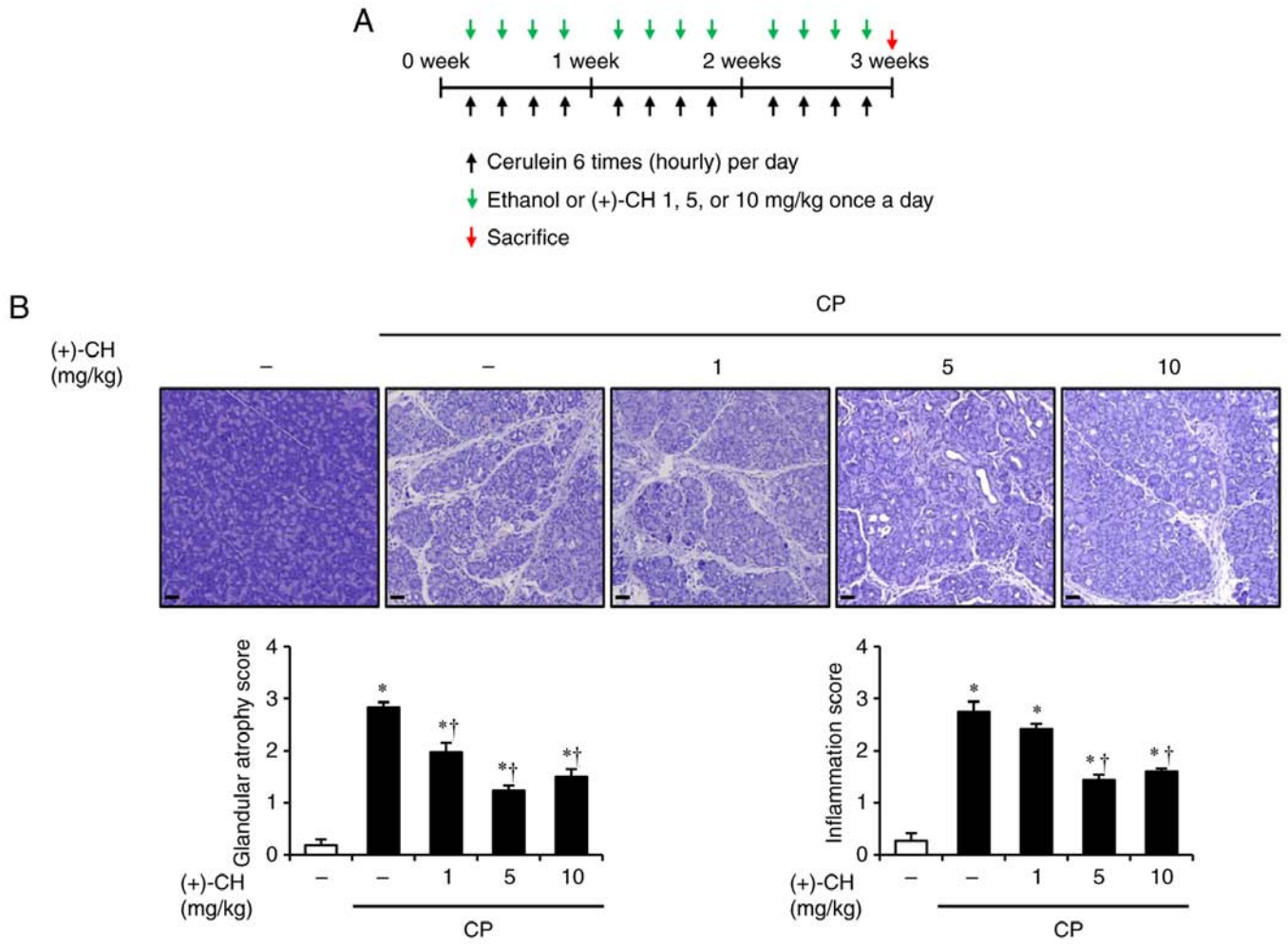

Figure 1. Effect of (+)-CH on histological damage in cerulein-induced CP. (A) Schematic depicting CP model in mice to examine the prophylactic effect of (+)-CH. (B) Representative hematoxylin & eosin-stained sections of the pancreas in the ethanol, CP and (+)-CH (1, 5 and 10 mg/kg) administered groups. Data are presented as mean ± SEM (n=3/group). Results are representative of three experiments. *P<0.05 vs. ethanol alone; †P<0.05 vs. CP alone. Scale bar, 50 μm. CP, chronic pancreatitis; CH, catechin hydrate.

Immunofluorescence (IF) staining. IF analysis of collagen I was performed using formalin-fixed pancreatic samples preserved in 25% sucrose. Unfrozen fresh pancreases were collected and fixed in 10% neutral-buffered-formalin solution (cat. no. HT501128; MilliporeSigma) for 24 h at RT. Following fixation, the specimens were embedded in FSC22 Frozen Section Media (cat. no. 3801480; Leica Microsystems GmbH) and cut into 9 μm thick sections. The tissues were blocked with 5% normal goat serum (Jackson ImmunoResearch Laboratories, INC.) at RT for 1 h and incubated with primary antibodies against collagen I (1:100; cat. no. ab34710; Abcam) diluted in 5% normal goat serum overnight at 4˚C. Subsequently, the tissues were incubated with fluorescence-labeled, Alexa Fluor® 488 goat anti-rabbit secondary antibodies (1:2,000; cat. no. A27034; Invitrogen; Thermo Fisher Scientific, Inc.) at RT for 2 h. Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI; 5 ng/ml; cat. no. D1306; Invitrogen; Thermo Fisher Scientific, Inc.) at RT for 5 min. The experiment was performed independently three times. The stained tissues were imaged using a FV1000 confocal laser scanning biological microscope (Olympus Corporation) at the Core Facility for Supporting Analysis and Imaging of Biomedical Materials at Wonkwang University (Iksan, Republic of Korea), supported by the National Research Facilities and Equipment Center (Daejeon, Republic of Korea). The samples were visualized using FV10-ASW 4.2 viewer (Olympus).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was isolated from the pancreas or pancreatic stellate cells (PSCs) using an Easy-Blue™ RNA extraction kit (cat. no. 17061; Intron Biotechnology, Inc.). RNA purity was verified using a Gene Quant Pro RNA calculator (Biochrom, Ltd.). RNA was reverse transcribed to cDNA using an ABI cDNA synthesis kit (cat. no. 4387406; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, the cDNA was amplified using Real-Time PCR SYBR Master Mix (cat. no. 4367659; Applied Biosystems; Thermo Fisher Scientific, Inc.) in a Step One Plus Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used in the present study were as follows: Acta-2 forward (F), 5'-GTCCCAGACATCAGG GAGTAA-3' and reverse (R), 5'-TCGGATACTTCAGCGTCA GGA-3'; fibronectin 1 (FN1) F 5'-GATGTCCGAACAGCT ATTTACCA-3' and R 5'-CCTTGCGACTTCAGCCACT-3'; Collagen I F 5'-GTGGTGACAAGGGTGAGACA-3' and R 5'-GAGAACCAGGAGAACCAGGA-3'; Collagen III F 5'-TAC

ACCTGCTCCTGTGCTTC-3' and R 5'-CATTCCTCCCAC TCCAGACT-3'; Collagen IV F 5'-CAAAGGCATCAGGGG AATAACT-3' and R (5'-ACCCTTAGATCCGTTGCATCC-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F 5'-TCCCACTCTTCCACCTTCGA-3' and R 5'-AGTTGG GATAGGGCCTCTCTTG-3'. Amplification was achieved by a series of steps as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min each, followed by dissociation for 15 s at 95˚C and 1 min at 60˚C, followed by 15 sec at 95˚C on ABI Step One Plus. Step One software v2.3 (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for data analysis. Relative gene expression (with target gene expression normalized to GAPDH) was calculated using the $2^{-\Delta\Delta Cq}$ method (31). The analysis was independently conducted thrice.

Isolation of PSCs. PSCs were isolated from C57BL/6 mice using the method previously described by Zang *et al* (32). Briefly, the pancreases of C57BL/6 mice were removed, minced with scissors and digested using collagenase-containing Gey's balanced salt solution (GBSS with sodium chloride; cat. no G9779; Sigma-Aldrich) for 20 min at 37˚C in a shaking water bath. Following collagenase digestion, the cell suspension was filtered through a 100 μm nylon mesh (cat. no. 352360; Falcon; Corning Life Sciences) and subjected to isopycnic separation with Nycodenz solution (Histonez; cat. no D2158; Sigma-Aldrich; Merck KGaA). Subsequently, the cells were collected from the top of the gradient, washed twice, resuspended in DMEM supplemented with 10% fetal bovine serum (cat. no. 16000-044; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (1 mg/5 ml; cat. no. 15140-122; Thermo Fisher Scientific, Inc.) and incubated in an environment with 95% O_2 and 5% CO_2. All experiments were performed by culturing cells between passages 0 and 3. PSCs were seeded at a density of $2x10^5$ cell/well in 6 well and $5x10^5$ cell/well in 6 cm dish. After confirming that (+)-CH did not exhibit any cytotoxic effects on PSCs at the specified concentrations, the following experiments were performed.

Cell treatment. To measure changes in the protein expression levels of Smad in TGF-β induced signaling, PSCs were pretreated with 250 μM (+)-CH for 1 h at 37˚C and then exposed to 0.5 ng/ml TGF-β1 (cat. no. 7666-MB; R&D Systems, Inc.) for 15 min at 37˚C. Whole cell lysates were harvested for further experiments. To provide information regarding the anti-fibrotic effect of (+)-CH at the mRNA level, cells were pretreated with (+)-CH at various concentrations (150, 200 and 250 μM) for 1 h at 37˚C and then stimulated with 0.5 ng/ml TGF-β1 or 25 ng/ml platelet-derived growth factor (PDGF)-BB (cat. no. 315-18; PeproTech, Inc.) for 24 h at 37˚C.

Western blotting. To acquire proteins from PSCs, the medium in 6 cm dishes with cells was discarded and cells were washed with ice-cold PBS. Cells were lysed with radioimmunoprecipitation assay lysis buffer (cat. no. IBS-BR004; iNtRON Biotechnology, Co.) containing 1% EZ block protease inhibitor cocktail (cat. no. K272-1; BioVision, Inc.) and 1% phosphatase inhibitor cocktail (cat. no. p5726; MilliporeSigma). The contents were agitated on ice for 1 h. The supernatants were boiled for 5 min at 99˚C in 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS; cat. no. S1377.0500; Duchefa Biochemie B.V.), 20% glycerol (cat. no. G6279; MilliporeSigma) and 10% 2-mercaptoethanol (cat. no. 21985-023; Thermo Fisher Scientific, Inc.). The BCA method was used for protein quantification. 20 μg proteins were separated on 8% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were then rinsed and blocked with 5% skim milk (cat. no. 232100; Becton, Dickinson and Company) in PBS containing 1% Tween 20 (cat. no. T9100-010; GenDEPOT, LLC) for 2 h at RT. The membranes were then incubated with diluted primary antibodies over night at 4˚C with gentle shaking. Antibodies against phosphorylated Smad2/3 (1:500; cat. no. 8828S; Cell Signaling Technology, Inc.), Smad2/3 (1:500; cat. no. 3102S; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 2118S; Cell Signaling Technology, Inc.) were used as the primary antibodies. After washing four times, the membranes were incubated in a diluted solution of goat anti-rabbit secondary antibodies (cat. no. SA002-500; GenDEPOT, LLC) for 1 h at RT. Antibodies were diluted with an immunoreaction enhancer solution (cat. no. NKB-101; Toyobo Life Science). The proteins were visualized using an enhanced chemiluminescence detection system (Amersham; Cytiva) according to the manufacturer's protocol. Samples were assessed in triplicate. Densitometric analysis of western blot was used by ImageJ version 1.53k (National Institutes of Health).

Statistical analysis. Data were analyzed using SPSS software (version 15; IBM, SPSS). Results are presented as the mean ± standard error of the mean. Significance was evaluated using a two-way analysis of variance (ANOVA) with time and dose parameters followed by post hoc Tukey's tests for multiple comparisons among groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of (+)-CH on biochemical parameters in mice. Toxicity testing is imperative in drug development. To assess the toxicity dosage of (+)-CH, biochemical parameters, ALP, ALT, AST, BUN and CREA, were assessed under ordinary conditions in mice. Either (+)-CH or ethanol (control) was administered intraperitoneally to the mice and serum samples were collected after 24 h to evaluate biochemical factors. Compared with the control group, neither (+)-CH nor ethanol demonstrated toxicity, even at 50 mg/kg (Table I). According to preliminary experiments, the effect of improving CP was better at a lower concentration (10 mg/kg) without toxicity (data not shown). Therefore, concentrations of (+)-CH <50 mg/kg were used in the subsequent experiments to determine its medicinal effects on CP.

Effect of (+)-CH on histological damage in cerulein-induced CP. CP leads to the destruction of the acini structure, which is replaced by fibrotic tissue (33,34). To determine whether (+)-CH could protect pancreatic acini from cerulein-induced destruction, pancreatic histological changes were examined. In response to repetitive inflammation, morphological features of the pancreas in CP demonstrated deformation of glandular

Table I. Biochemical parameters of mice treated with (+)-CH in acute toxicity test.

Group	ALP, IU/l	ALT, IU/l	AST, IU/l	BUN, mg/dl	CREA, mg/dl
Normal	293.67±46.37	26.33±1.53	144.33±46.50	21.33±3.51	0.11±0.02
Ethanol	277.67±18.77	20.67±3.21	130.00±33.42	21.00±1.00	0.12±0.01
(+)-CH, mg/kg					
1	253.33±18.77	20.67±0.58	160.67±6.66	23.33±3.21	0.11±0.00
10	246.67±9.24	23.00±1.00	157.00±6.56	16.33±2.31	0.08±0.01
20	292.33±37.21	26.33±4.16	152.67±14.74	19.00±1.73	0.08±0.01
50	235.00±15.87	25.33±2.31	147.33±8.96	16.00±2.65	0.07±0.02

CH, catechin hydrate; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; CREA, creatinine.

tissues, characterized by loss of acinar cells and transition to acinar-to-duct metaplasia (Fig. 1B). In contrast, the group administered (+)-CH at concentrations of 1, 5 and 10 mg/kg revealed slight edema and acinar cell transformation, which suggested mild glandular atrophy. The infiltration of immune cells in the (+)-CH groups was markedly lower than that in the CP group (Fig. 1B).

Effect of (+)-CH on the activation of PSCs and production of ECM components during CP. α-SMA and extracellular matrix (ECM) proteins are highly expressed in peri-acinar fibrotic areas during CP development (35). α-SMA is regarded as a marker of the activated PSCs (36). α-SMA was stained and the mRNA expression level of *Acta-2* was assessed to investigate the PSC activation regulatory ability of (+)-CH in CP. In CP tissue, α-SMA was stained strongly with DAB in the vicinity of the damaged acini but was expressed at significantly lower levels in tissues in the (+)-CH-administered groups (Fig. 2A). The mRNA expression of *Acta-2* in the CP group was considerably higher than that in the control group and it was suppressed in the (+)-CH group (Fig. 2B). During fibrogenesis, ECM protein levels increase substantially in the fibrotic region and are often accompanied by morphological alterations (35,36). To assess the preventive effect of (+)-CH on the production of ECM components, IF staining and RT-qPCR were performed. Unlike in the normal pancreas, the structure of type I collagen (Col I) in the CP tissue was observed to be irregular and thicker and the staining (green) intensity was also stronger than that of samples from the normal pancreas. However, overall alterations and Col I deposition decreased significantly in the pancreas of mice pretreated with (+)-CH at concentrations of 5 and 10 mg/kg (Fig. 2C). To clarify these results, the mRNA expression levels of *FN1* and different types of collagens were evaluated. In particular, (+)-CH concentrations of 5 and 10 mg/kg significantly reduced the mRNA expression levels of *FN1* and *Col I* compared with the control. In contrast, the decrease in type III (*Col III*) and type IV (*Col IV*) collagens, compared with the control, was significant only at a concentration of 5 mg/kg (Fig. 2D and E). As 5 mg/kg (+)-CH demonstrated greater effect than the other concentrations used for the determination of prophylactic effects of (+)-CH on CP, 5 mg/kg (+)-CH was considered the optimal concentration and further experiments were performed using this concentration.

Effect of (+)-CH on the activation of isolated PSCs. Activated PSCs are involved in pancreatic fibrogenesis and are the principal source of α-SMA and ECM proteins (36,37). Adjusting the activation of cultured PSCs parallels the regulation of persistent activation of PSCs *in vivo*, which is a critical event in fibrosis (37). Transforming growth factor-β (TGF-β) has been reported to mediate continuous activation of PSCs, and Smad is part of one of the TGF-β signal transduction pathways (37,38). As TGF-β drives PSC activation signal through a Smad2-dependent pathway (39), the protein expression of phosphorylated Smad2 and total Smad2 was assessed to evaluate whether (+)-CH regulated TGF-β signaling. It was demonstrated that (+)-CH significantly inhibited the activation of Smad2 after TGF-β1 treatment in cultured PSCs (Fig. 3A). Furthermore, the regulatory effects of fibrotic factors that appear as a result of the TGF-β cascade were assessed. Treatment with TGF-β1 significantly decreased *Acta-2*, *FN1*, *Col III* and *Col IV* mRNA expression levels at (+)-CH concentrations of 150, 200 and 250 μM compared with the control (Fig. 3B). Moreover, the mRNA expression level of *Col I* was significantly reduced only at a concentration of 250 μM (+)-CH. Changes in the mRNA expression levels of *Acta-2* and ECM components in the PSCs exposed to platelet derived growth factor (PDGF), contribute to the mitogenic effect of PSCs (40). Treatment with (+)-CH significantly decrease the mRNA expression levels of *Acta-2*, *FN1*, *Col I*, *Col III* and *Col IV* compared with the PDGF-BB-only treatment group (Fig. 3C).

Therapeutic effect of (+)-CH on CP. Based on earlier findings, whether (+)-CH demonstrated a therapeutic effect against CP was evaluated. Treatment with (+)-CH for 1 or 2 weeks alleviated glandular atrophy and inflammation during CP (Fig. 4). Treatment with (+)-CH for 2 weeks resulted in faint staining for α-SMA. However, treatment with (+)-CH for 1 week resulted in a staining as strong as that observed for the CP group (Fig. 5A). The mRNA expression level of *Acta-2* decreased markedly when (+)-CH was administered for 1 week and was significantly decreased in the group with (+)-CH administered for 2 weeks, compared with the control (Fig. 5B). As for α-SMA, the deposition of Col I and mRNA expression levels of *FN1*, *Col I*, *Col III* and *Col IV* were all significantly reduced in the group injected with (+)-CH for 2 weeks, compared with the control (Fig. 5C-E).

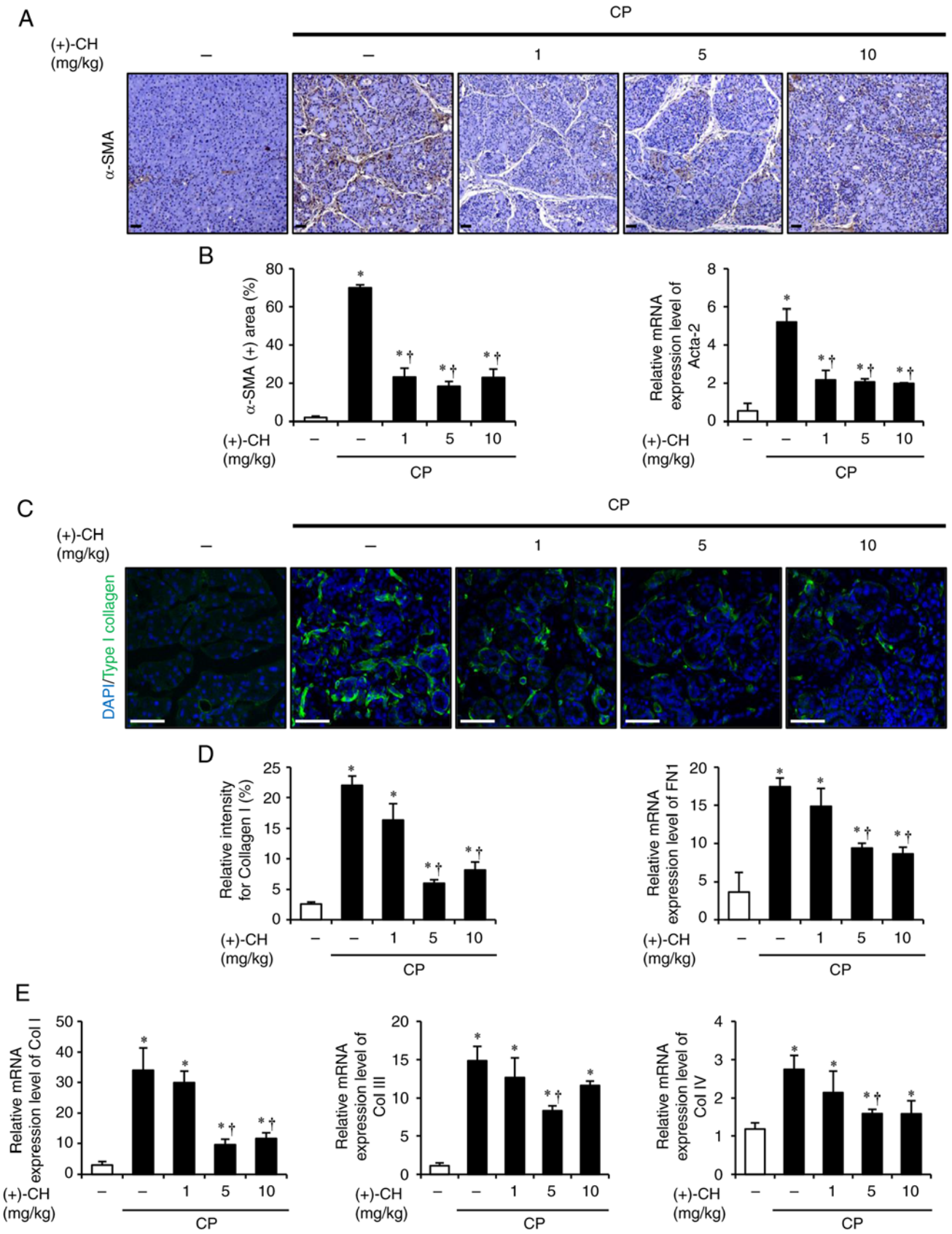

Figure 2. Effect of (+)-CH on PSC activation and production of ECM components during CP. (A) Images of 3,3'-diaminobenzidinestaining for α-SMA in the ethanol, CP and (+)-CH (1, 5 and 10 mg/kg) groups; brown color indicates α-SMA$^+$ cells. (B) The mRNA expression level of *Acta-2* in the pancreas was assessed using RT-qPCR. (C) Confocal images of immunofluorescence staining for collagen I in the ethanol, CP and (+)-CH (1, 5, 10 mg/kg) groups; green color indicates collagen I$^+$ cells and blue color indicates DAPI$^+$ cells. (D) mRNA expression of *FN 1* in the pancreas was assessed using RT-qPCR. (E) The mRNA expression levels of *col I*, *III* and *IV* in the pancreas were assessed using RT-qPCR. Data are presented as mean ± SEM (n=3/group). Results are representative of three experiments. *P<0.05 vs. ethanol alone; †P<0.05 vs. CP alone. Scale bar, 50 μm. CP, chronic pancreatitis; CH, catechin hydrate; PSC, pancreatic stellate cell; ECM, extracellular matrix; α-SMA/Acta-2, α-smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; RT-qPCR, reverse transcription-quantitative PCR; FN1, fibronectin 1; Col, collagen.

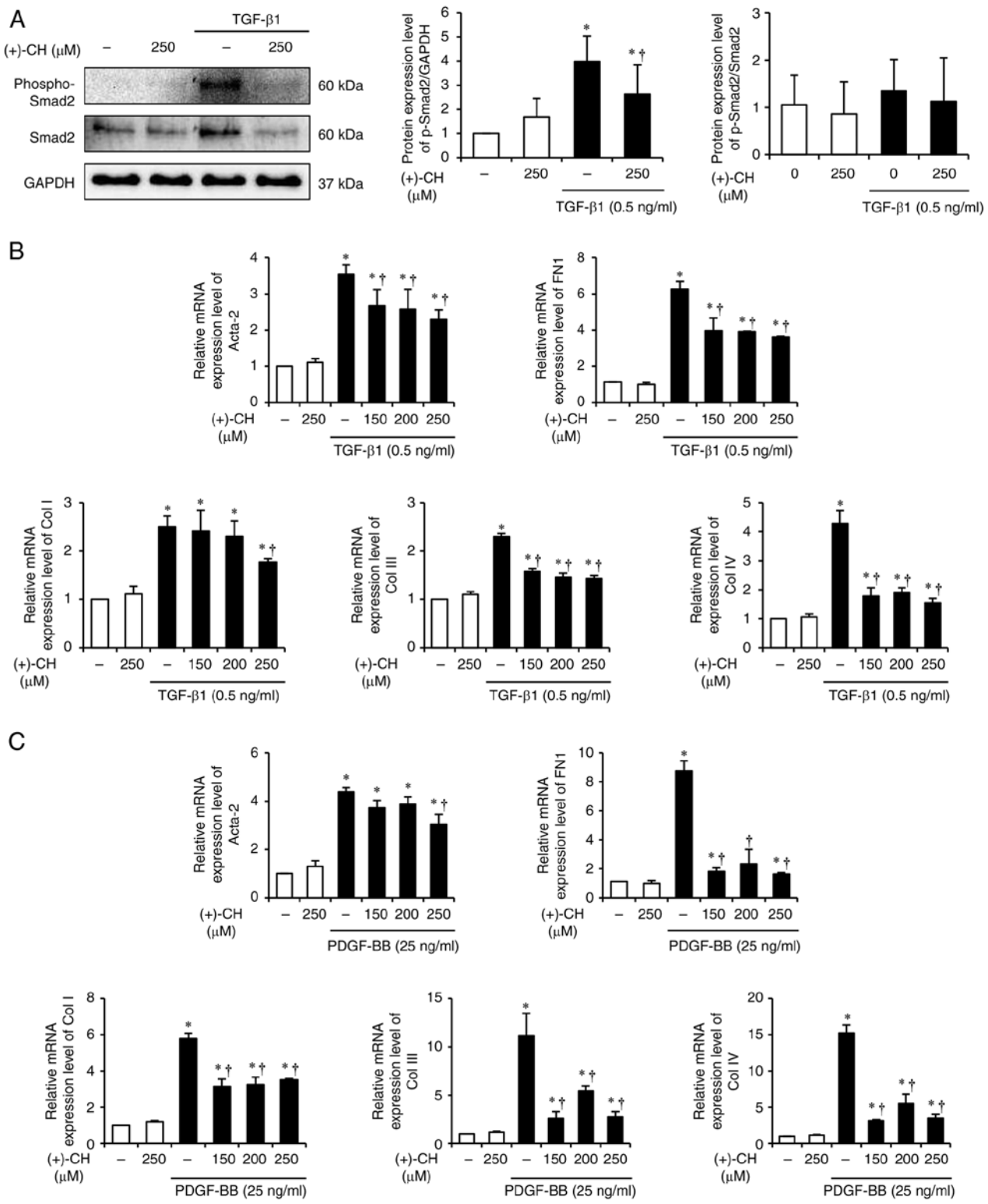

Figure 3. Effect of (+)-CH on PSC activation in isolated PSCs. (A) Protein expression levels of p-Smad2 (Ser465/467) and Smad2 were assessed using western blotting; GAPDH was used as the loading control. (B) The mRNA expression levels of TGF-β1 induced *Acta-2*, *FN1* and *col, III* and *IV* in isolated PSCs were assessed using RT-qPCR. (C) The mRNA expression levels of PDGF-BB induced *Acta-2, FN 1* and *col I, III* and *IV* in isolated PSCs were assessed using RT-qPCR. Data are presented as mean ± SEM. Results are representative of three experiments. *P<0.05 vs. ethanol alone; †P<0.05 vs. TGF-β1 alone or PDGF-BB alone. CP, chronic pancreatitis; CH, catechin hydrate; phospho-Smad2/pSmad2, phosphorylated Smad2; Col, collagen; Acta-2, α-smooth muscle actin; FN1, fibronectin 1; PDGF-BB, platelet-derived growth factor; p, phosphorylated; RT-qPCR, reverse transcription-quantitative PCR; FN1, fibronectin 1; Col, collagen.

Discussion

CP is a pancreatic inflammatory disease accompanied by fibrosis and can develop at any life stage from children to adults, thus greater attention should be paid to it (41). CP is difficult to diagnose in the early stages of the disease due to ambiguous symptoms such as weight loss, pain and steatorrhea, something which further impedes the quality of life

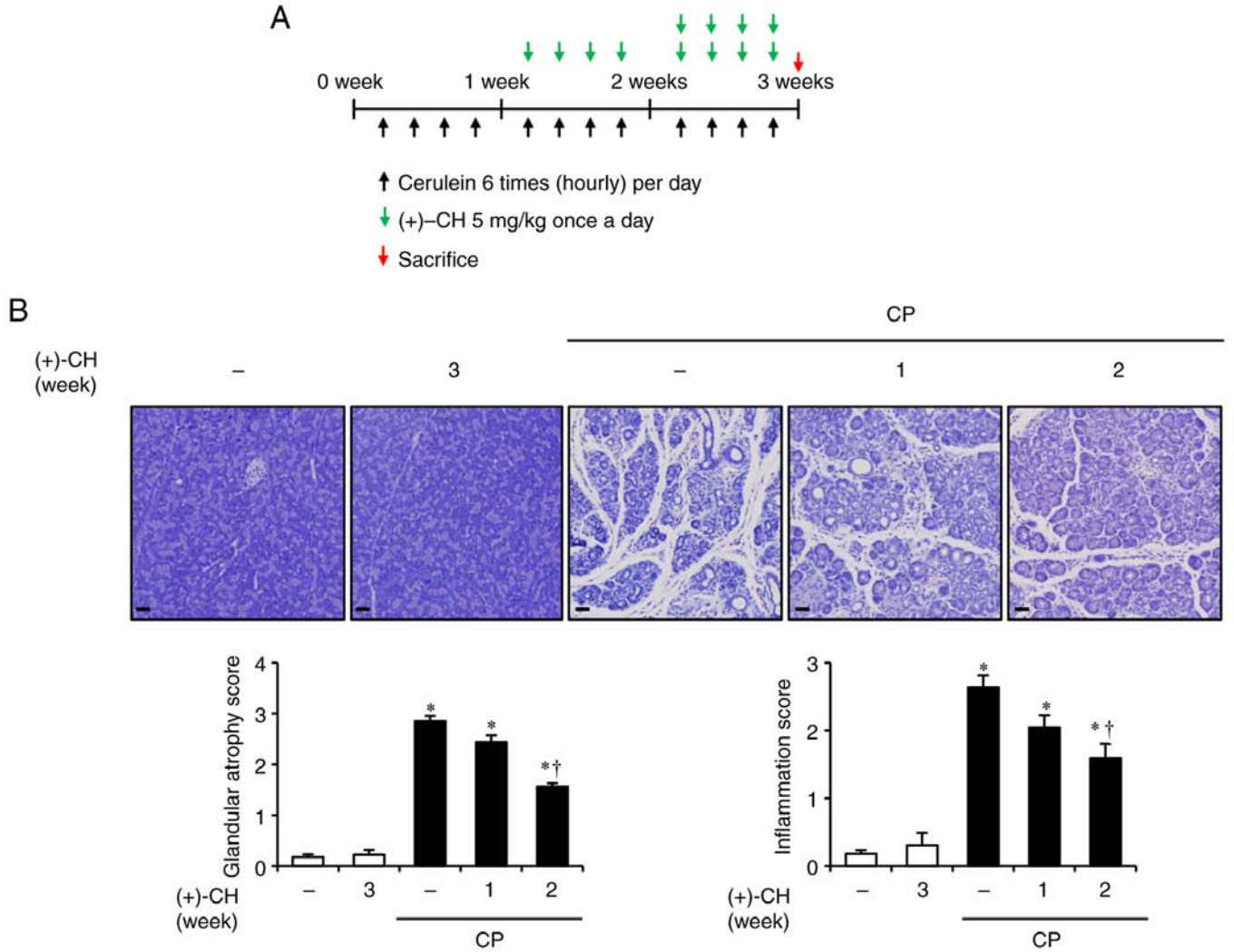

Figure 4. Therapeutic effect of (+)-CH on CP. (A) Schematic depicting the CP model used in mice to investigate the therapeutic effect of (+)-CH. (B) Representative hematoxylin & eosin-stained sections of the pancreas in the ethanol, (+)-CH alone, CP and 5 mg/kg (+)-CH groups (1 and 2 weeks). Data are presented as mean ± SEM (n=3/group). Results are representative of three experiments. *P<0.05 vs. ethanol alone; †P<0.05 vs. CP alone. Scale bar, 50 μm. CP, chronic pancreatitis; CH, catechin hydrate.

of patients (3,42). Therefore, understanding the process of pancreatic fibrogenesis and finding a way to treat it are considered primary objectives (3).

The progression of CP includes irregular distortion of the glandular parenchyma owing to the formation of a duct-like structure following pancreatic inflammation, which results in pancreatic exocrine and endocrine loss (43). Destruction of the pancreatic acini leads to impairment of pancreatic function and pain, which are clinical features of CP (44). Inflammatory infiltrates, which are increased in the pancreas of patients with CP, affect the proliferation and activation of PSCs by releasing cytokines and stimulating collagen synthesis to promote pancreatic fibrosis (45,46). As glandular tissue destruction and necrosis are essential prerequisites of fibrotic pancreatic tissues (34), the histological changes, including acini atrophy and immune cell infiltration in pancreatic tissues, after (+)-CH injection were assessed and it was demonstrated that (+)-CH alleviated parenchymal destruction and inflammation at all tested concentrations.

The main pathological feature of CP is pancreatic fibrosis and PSCs, resident cells in the inter-acinar regions, contribute to the pathogenesis of CP in an activated state by acting as key effector cells in fibrogenesis (37,47,48). Activated PSCs exhibit morphological changes, including α-SMA expression and production of ECM proteins such as collagen and FN (37). α-SMA, which is abundantly observed in pancreatic fibrotic areas, is the first to be expressed in the process of fibrosis and is considered a marker of activated PSCs (38). During fibrogenesis, acinar cells are destroyed and replaced with connective tissue, which arises from increased or disorganization of deposited ECM proteins (35). FN, which is an omnipresent non-collagen ECM component, participates in a variety of cellular processes, including proliferation and migration; excessive deposition of FN leads to organ impairment, which results in fibrotic diseases (49,50). Overexpression of FN1, which belongs to the FN family, precedes collagen deposition and is often considered an indicator of collagen accumulation (46,51). Col I and Col III, which are the main collagens constituents of the interstitial matrix, are upregulated in a disorganized state with FN in CP tissue and are formlessly deposited in thick bundles (36). Col IV, which

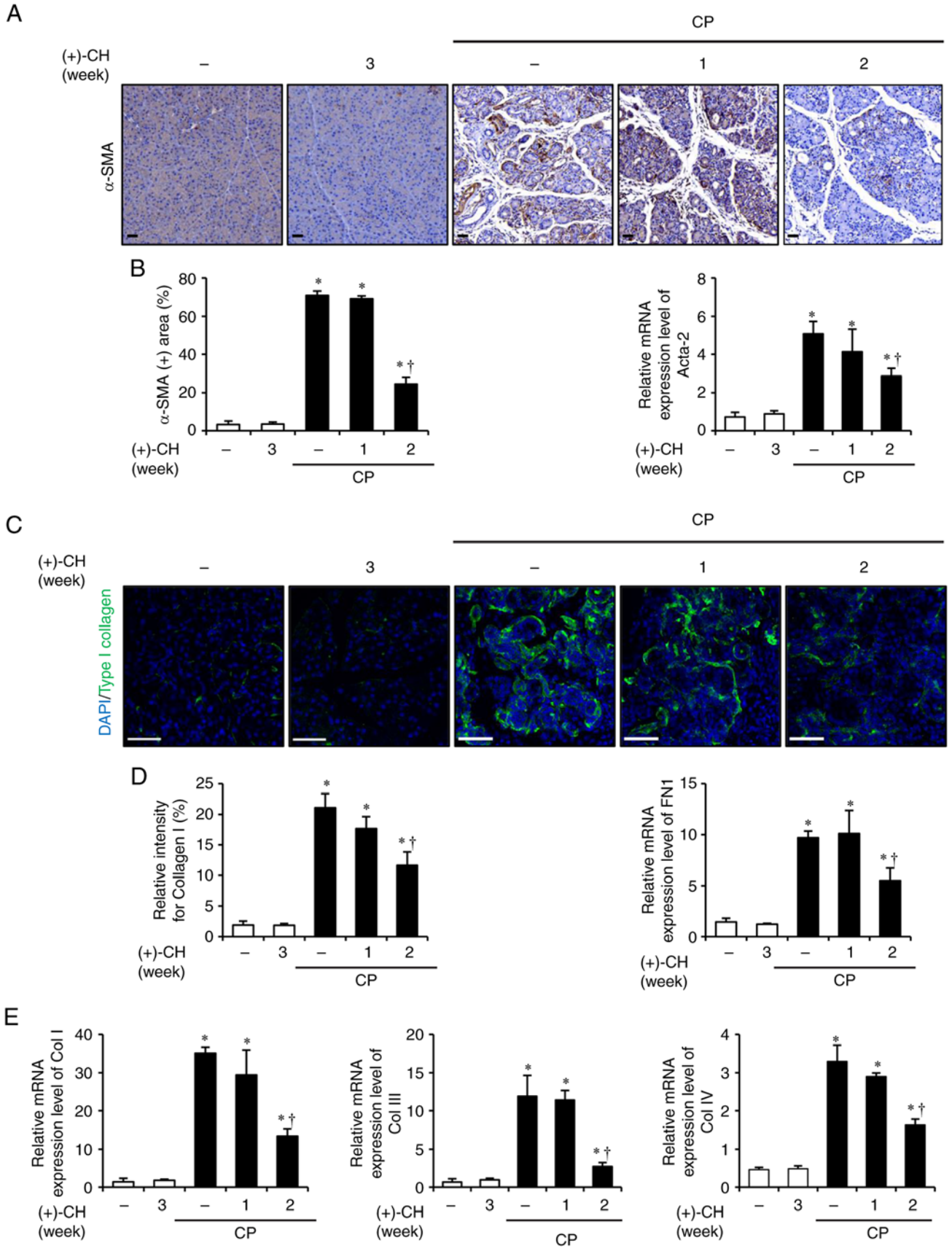

Figure 5. Therapeutic effect of (+)-CH on pancreatic fibrosis. (A) Images of 3,3'-diaminobenzidine staining for α-SMA in the ethanol, (+)-CH alone, CP and 5 mg/kg (+)-CH groups (1 and 2 weeks); brown color indicates α-SMA$^+$ cells. (B) mRNA expression level of *Acta-2* in the pancreas was assessed using RT-qPCR. (C) Confocal images of immunofluorescence staining for collagen I in the ethanol, (+)-CH alone, CP and 5 mg/kg (+)-CH groups (1 and 2 weeks); green color indicates collagen I$^+$ cells and blue color indicates DAPI$^+$ cells. (D) mRNA expression level of *FN1* in the pancreas was assessed using RT-qPCR. (E) mRNA expression levels of *col I, III* and *IV* in the pancreas were assessed using RT-qPCR. Data are presented as mean ± SEM (n=3/group). Results are representative of three experiments. *P<0.05 vs. ethanol alone; †P<0.05 vs. CP alone. Scale bar, 50 μm. CP, chronic pancreatitis; CH, catechin hydrate; α-SMA/Acta-2, α-smooth muscle actin; FN1, fibronectin 1; RT-qPCR, reverse transcription-quantitative PCR; col, collagen.

is categorized as a network-forming collagen, is located in the basement membrane (BM), which binds to numerous factors and is responsible for forming the backbone of the BM (52). During acinar cell atrophy, Col IV accumulation varies and is discontinuous in fibrotic areas adjacent to the BM (50). Given that collagen production increases in activated PSCs during fibrogenesis (38), the inhibitory effect of (+)-CH on the activation of PSCs and synthesis of ECM proteins was demonstrated in the present study. The upregulated expression of α-SMA (also known as Acta2), collagens and FN1 and (+)-CH was demonstrated to have significantly prevented the activation of PSCs and collagen deposition compared with the CP group. These results indicated that (+)-CH has a prophylactic effect on CP.

Following stimulation, the acinar cells are injured, which results in pancreatic inflammation. As a consequence of pancreatic injury, paracrine factors such as cytokines, growth factors and intracellular signaling molecules are secreted by the injured cells (37,53). In response to the paracrine factors, PSCs are persistently activated and subsequently regulate the proliferation, migration and differentiation of PSCs, thereby inducing fibrogenesis (37,53). Among autocrine and paracrine factors, the growth factors PDGF and TGF-β are the major factors responsible for the development of pancreatic fibrosis (38). TGF-β is secreted mainly in damaged acinar cells adjacent to fibrotic areas and is a potent promoter of PSC activation (38). Smads, which have been implicated in the fibrotic effect of TGF-β, can be directly phosphorylated by TGF-β receptors (54). Phosphorylated receptor-regulated Smads form a complex with the common mediator Smad and translocate into the nucleus, thereby regulating the transcription of target genes (54). TGF-β not only increases α-SMA expression and collagen synthesis but also enhances PDGF receptor expression, thereby increasing the responsiveness of PSCs to PDGF, resulting in continuous PSC activation and permanent fibrosis in the pancreas (40). PDGF-BB has been reported to have shown a proliferative effect demonstrated by increased cell numbers and DNA synthesis in PDGF-BB-exposed PSCs (40). The cell proliferative effect of PDGF is also affected by upregulated cell surface PDGF receptor in fibrotic areas (40). In cultured PSCs, (+)-CH inhibited Smad2 activity caused by TGF-β and suppressed production of fibrosis factors. Likewise, the levels of fibrosis factors acting through PDGF were significantly reduced in PSCs treated with (+)-CH.

According to the European Food Safety Authority (Parma, Italy), green tea catechins, particularly EGCG, are generally considered safe if the amount adheres to the dosage recommended by European member states (23). In addition, (+) catechin is classified as having the least toxicity compared with other catechins, including EGCG and epicatechin gallate (ECG), in normal HGF-2 fibroblasts (25). To date, no specific toxicity has been reported for (+) catechin or (+) CH. An inhibitory effect of EGCG on PSC activation has been reported previously (19). However, there are few studies on the protective effects of (+) catechin or (+) CH against pancreatic fibrosis. Because existing CP treatment, such as pregabalin can result in adverse side effects include dizziness, somnolence, blurred vision, even fainting (55,56), (+) catechin or (+)-CH was anticipated to be an improved therapeutic agent. Therefore, the therapeutic effect of (+)-CH following its prophylactic effect against CP was assessed. The severity of pancreatic tissue damage and fibrosis exhibited in CP improved when (+)-CH was administered for 2 weeks. These results provided evidence to support the hypothesis that (+)-CH has a therapeutic effect on CP, nonetheless, further studies pertaining to the analysis of safety and efficacy of (+)-CH are needed.

In conclusion, the findings of the present study demonstrated that (+)-CH inhibited immune cell infiltration and histological injury of the pancreas. Moreover, the administration of (+)-CH contributed to a reduction in the levels of fibrosis-related factors, including Acta-2, FN1, Col I, Col III and Col IV both in TGF-β1 and PDGF-BB treated PSCs, and in CP. These results demonstrated that (+)-CH had prophylactic and therapeutic effects on CP. (+)-CH protected against the progression of pancreatic fibrosis by inactivating the TGF-β/Smad2 signaling pathway. Therefore, the results of the present study suggest that (+)-CH itself or (+)-CH containing products may be a novel therapeutic strategy for the treatment of CP. However, as efficacy was confirmed only in cerulein induced CP and the safety issue of catechins, further studies are required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BK and DUK validated the data, performed the experiments, wrote the original draft of the manuscript. JYO performed the experiments. GSB and SJP conceptualized and supervised the study and performed project administration. BK, DUK, JYO, GSB and SJP confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed in accordance with the animal care regulations set forth and approved by The Wonkwang University Animal Ethics Committee (Iksan, Republic of Korea; approval no. WKU22-43).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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