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Anti-inflammation and anti-fibrosis with PEGylated, apigenin loaded PLGA nanoparticles in chronic pancreatitis disease

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ABSTRACT

Chronic pancreatitis (CP) is a disease that imposes great suffering symptoms (chronic abdominal pain, diabetes, impaired digestion and high risk of pancreas cancer, etc.) to people’s life. However, treatment of CP is limited by the lack of effective therapies. In this work, apigenin, a drug that can inhibit pancreatic stellate cell fibrosis, is loaded into PEGylated PLGA nanoparticles to treat CP. The nanoparticles have a diameter around 160 nm, and loading efficiency of 96 µg apigenin per mg PLGA nanoparticles. Our studies find that apigenin loaded nanoparticles can inhibit PSCs growth and promote PSCs apoptosis. To investigate the mechanism of apigenin in treating CP, PSCs with pro-inflammatory and pro-fibrotic response are treated with apigenin loaded nanoparticles, and analyzed with RT-PCR. Our study finds that apigenin loaded nanoparticles can reduce the expression of mRNAs associated with PSC pro-inflammation and pro-fibrosis (e.g. collagen 1A1 mRNA, fibronectin mRNA and IL-6 and IL-8 mRNAs). *In vivo* study finds that apigenin loaded in PEGylate PLGA nanoparticles have a longer circulation time in mice than apigenin in free form. Consistent with *in vitro* study, *in vivo* study also confirms the anti-inflammation and anti-fibrosis effect of PEGylated, apigenin loaded PLGA nanoparticles by reducing the inflammation and fibrosis associated mRNAs.

KEY WORDS: Chronic pancreatitis, pancreatic stellate cells, apigenin, PEGylated PLGA nanoparticles, pro-inflammatory and pro-fibrosis mRNAs, *in vivo*
INTRODUCTION

Chronic pancreatitis (CP) is a disease featured by chronic inflammation, glandular necrosis, and fibrosis.\(^1,2\) People have chronic pancreatitis suffer from extremely uncomfortable symptoms including chronic abdominal pain, impaired digestion, malnutrition, anorexia, diabetes et... Of note is that studies have found that patients with CP have a much higher risk of developing pancreatic cancer, which has a very low five year survival rate.\(^3,4\) Despite these sufferings and risk, current treatment of CP is only limited to some supportive cares or symptom palliation (e.g. pain management, digestive enzyme replacement etc...), with merely therapy available for effective treatment. Chronic pancreatitis is usually associated with physiological symptoms including inflammatory response from immune cells, focal necrosis/fibrosis, and auto-digestion of relevant tissues.\(^5,6\)

Epidemiological, etiological and experimental studies have been performed to investigating the causes of chronic pancreatitis. Multiple risk factors contribute to the development of CP, including alcohol consumption, smoking, genetic predisposition, immunological reasons, disease related to metabolism. Accumulated data indicates chronic pancreatitis is a result of damage incurred during repeated bouts of acute pancreatitis, which results in chronic inflammation microenvironment with irreversible fibrosis.\(^7,1\) In particular, fibrotic scars usually develop from repeated injury in pancreatic tissue, where insufficient exocrine and endocrine are secreted. Pancreatic stellate cells (PSCs) are the major cells that are in charge of chronic pancreatitis scaring. In chronic pancreatitis, PSCs are activated and proliferated, followed by migrating to injury sites and producing extracellular matrix proteins and cytokines that induce inflammation and fibrosis of pancreas.\(^8,9\) The inhibition over inflammation and fibrosis associated with PSCs is therefore a key step in CP treatment.

Apigenin (4’, 5, 7-trihydroxyflavone) is a flavonoid that exists in herbs like chamomile tea.\(^10\) Recent studies find that apigenin possess great potential in anti-inflammation,\(^11\) anti-proliferation.\(^12\) In addition, apigenin has potential uses in cancer prevention and therapy, and it suppresses cell growth against many human cancer cell lines, including breast, colon, skin, thyroid, leukemia, and prostate cancer cells. Particularly, recent studies find that apigenin can protect the pancreas from repeated pancreatic injury by inducing the apoptosis of PSCs.\(^13\) However, the potential use of apigenin for CP treatment is limited by its extremely low solubility and bioavailability.
In this study, we employ PEGylated poly(lactic-co-glycolic acid) (PLGA) nanoparticles, a state-of-art material for drug delivery,\textsuperscript{14, 15} to improve the delivery efficiency and bioavailability of apigenin for CP treatment. Considering the significant challenge that is current no active target for CP treatment,\textsuperscript{16, 17} the particles surface was modified with PEGylation to enhance its \textit{in vivo} circulation time and delivery efficiency. Study at cellular level find that apigenin loaded nanoparticles can inhibit PSCs proliferation and induce PSCs apoptosis. Study at molecular level finds apigenin loaded nanoparticles inhibit chronic pancreatitis development by inhibiting collagen 1A1 mRNA, fibronectin, IL-6 and IL-8 mRNAs in activated PSCs cells. Our \textit{in vivo} study indicates that apigenin loaded in PLGA has a longer circulation time in mice and reduce the production of pro-fibrosis and pro-inflammation mRNAs in mice PSC cells.

**MATERAIL AND METHODS**

**Materials:** Penicillin, streptomycin, amphotericin, and gentamicin were from InvivoGen. Apigenin was obtained from Sigma Aldrich. Dulbecco’s Modified Eagle Medium (DMEM) was from Shanghai Baolai Biotech. Non-essential amino acid was from VWR. Fetal bovine serum (FBS) was from VWR. Amine-terminated methoxypolyethylene glycol (mPEG-NH2, MW = 5000) was from Sigma. EZ-Link Sulfo-NHS-LC-Biotin was purchased from Life Technologies. Target retrieval solution and antibody diluent was from Shanghai BlueGene Biotech. Liquid 3, 3’-diaminobenzidine (DAB), and substrate chromogen system was from Sigma. Poly (lactic-co-glycolic acid) (PLGA, lactide:glycolide (50:50), MW=30,000-60,000) was from Sigma. Fibronectin antibody, pronase, collagenase, DNAse and Gey’s balanced salt solution were purchased from Shanghai BlueGene Biotech. Biotinylated secondary antibody, VECTASTAIN Elite ABC kit, and VectaMount were from Vector Laboratories, Inc. Hematoxylin 7211 counterstain was from Thermo Fisher Scientific Inc. Human parathyroid-related protein (1-36) was obtained from PolyPeptide Laboratories (San Diego, CA); 4, 6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich. Fibrillar acidic protein was from Shanghai Blue Gene Inc.

**Synthesis and characterization of surface modified, apigenin loaded nanoparticles:** Surface modified, apigenin loaded nanoparticles were synthesized by single emulsion. Surface modification of nanoparticles was performed according to what reported in literature.\textsuperscript{18, 19} Briefly, 20 mg of apigenin and 100 mg of PLGA were dissolved in
2 ml DCM as organic phase. The organic phase was sonicated for 45 s and added drop-wise into 2 mL of PVA (2%) + 2 ml avidin-palmitate conjugate solution. After sonicating for 30 s, the mixture was stirred with magnetic bar at 800 rpm for 2 h. The nanoparticles were collected by centrifuging at 12500 rpm for 5 min, followed with two times of washing with DI water. The nanoparticles were filtered through a 40 µm size strainer to remove the extra-large particles, followed by lyophilization and storage at −20°C before use. Surface modification of nanoparticles with PEG-biotin was performed before use. Briefly, the nanoparticles were in incubated in 20 mg/ml PEG in PBS for 15 min at room temperature. Surface modified and unmodified nanoparticles were imaged under scanning electron microscope (SEM, JEOL- JSM 7500F Scanning Electron Microscope). Size and zeta potential of nanoparticles were measured with Zetasizer Nano ZS (Malvern). The incident beam was scattered and detected at an angle of 90° at the average of 12 runs with triplicate for each run. Zeta potential of nanoparticles was assessed by the same equipment by following the same procedure.

Chronic pancreatitis (CP) mice model: All the animal studies mice were approved ethical committee board for animal experiment in Huzhou University. The model was established according to what reported in literature and was slightly modified. Briefly, cholecystokinin analog was injected to mice (C57/BL6 mice, female, 4-8 week old) every five hours for 3 weeks. The injections resulted in aberrant secretion of zymogen, and premature activation of PSCs. Histologic and pathophysiological features of CP were generated from the inflammation induced by the repeated injury.

Mice treatment: After inducing CP in mice, the mice were treated with apigenin in free form or apigenin loaded in nanoparticles (96 µg apigenin in nanoparticles) or empty nanoparticles once per day. Pancreases of all mice were collected after 3 weeks of treatment.

Cell isolation and culture: To isolate PSC, pancreases were dissected from mice, and minced into pieces with a size smaller than 1 mm³, followed by incubation in solution that contained 0.02% pronase, 0.05% collagenase P, and 0.1% DNAse in Gey's balanced salt solution (GBSS) for 25 min. The minced tissue was then piped up and down with 10 ml GBSS with 0.3% BSA and 10 ml of 28.7% (wt/vol) of Nycodenz in Gey’s solution without adding any salt. The whole solution was then centrifuged at 1000g for 30 min, where cells were located into a fuzzy band on top of the interface of the Nycodenz cushion and GBSS. After collecting the cells from cell
bands, cells were resuspended in Iscove’s modified Dulbecco’s medium enriched with fetal calf serum (10%), glutamine (4 mM), and antibiotics (penicillin 100 units/ml; streptomycin 100 µg/ml). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. The successful isolation of PSCs in was confirmed by staining the cells for vimentin as well as glial fibrillary acidic protein.

**Proliferation and viability assays:** For proliferation assays, PSCs were cultured in 6 well plates at a density of 4 x 10⁵ cells per well. The cells were treated with apigenin loaded nanoparticles or PBS or apigenin in free form for certain amount of time, with the number of cells counted with cell counter. Viability of cells are assessed with trypan blue (5 µM) in PBS, with viability of cells assessed by a cell counter (LUNA™ Automated Cell Counter).

**Reverse transcription-polymerase chain reaction (RT-PCR) assay:** A RNA aqueous kit (Invitrogen TRIzol Reagent) was used to isolate RNA from PSCs. The operations were conducted by following manufacturer’s instructions. Nanodrop (Thermo Scientific NanoDrop 2000 Spectrophotomete) was used to quantify RNA concentration. TaqMan Reverse Transcription Reagents Kit (Life Technologies) was used to generate DNA by using 1 mg RNA. SYBR-green (Roche) was used for RT-PCR, with the following primers employed: for mice collagen type 1α1, forward primer GGCAGCCTCTCTGATTTCTG and reverse primer CTTGGCAAAACTGCACCTTCA were used; For mice fibronectin, forward primer ATGGTGTGAGATACAGTGGCTACTG and reverse primer TCGACAGGACCACCTTGAGCTT were used; For mice collagen type 1α forward primer GGCAGCCTCTCTGATTTCTG and reverse primer CTTGGCAAAACTGCACCTTCA were used. For mice IL-6, forward primer ATGAACTCCTTCTCCACAAGCG and reverse primer CCCCAGGGAGAAGGCAAC were employed; For mice IL-8, forward primer GGCAGCCTTCTCCTGATTTCTG and reverse primer CTTGGCAAAACTGCACCTTCA were used. 18S levels were used to normalize each gene target, where the relative expression was calculated by: n-fold change = 2⁻¹K, with K= (C₇(target)-C₇(sample)).

**Immunohistochemistry staining:** The following procedures were performed for immunohistochemical staining:²²,¹³ pancreases were fixed in formalin (10%, v/v) for 72 h at 4°C. The tissues were then embedded in paraffin blocks, sliced into a thin layer with a thickness of 4 mm, and attached onto glass substrate. The glass
substrate was then incubated in 10 mM citrate buffer (pH=6.3) for 45 min at 97 °C, and then washed twice with DI water. Hydrogen peroxide at a concentration of 3% (v/v) was used to inhibit endogenous peroxidase for 20 min at room temperature. Rabbit serum and bovine serum albumin dissolved in PBS (3% wt/wt for each solution) were used to incubate the samples for 3 h at room temperature to prevent non-specific binding of antibodies. The samples were then incubated with rabbit anti-IG 4 (600x diluted) for 16 h at 4°C, followed by washing with 0.1% Tween 20 for 5 min, and then washed with PBS for 5 min for twice. The samples were then incubated with biotinylated rabbit anti-goat (400x diluted) for 30 min at room temperature, followed by washing with 0.1% Tween 20 and PBS for 5 min. Each washing step was repeated twice. VECTASTAIN Elite ABC kit was used to stain fibronectin. After washing the samples with DI water for 3 min, hematoxylin 7211 was used to stain the samples for 3 min, followed by dehydrating the glass substrate with ethanol and xylene. Zeiss Light & Fluorescence Microscope was used for imaging. Image J was used to quantify the fibronectin staining.

*In vivo* bio-distribution of nanoparticles: Apigenin, either in free form or loaded in nanoparticles, were injected to female Balb/C mice (6–8 weeks old) through tail vein, followed by sacrificing the mice with CO₂ at certain time point (12 to 72 h) after injections. Blood at a volume of 6-10 ml was collected though cardiac puncture. Plasma was isolated from blood by centrifugation (16000G, 5min). The plasma collected was diluted by extraction buffer (10% triton X-100: acidified isopropanol (0.75 M HCl): DI water = 1:2:15, volume ratio) at a volume ratio of 1:10, and stored at −20 °C for 24 h.

**RESULTS**

**Synthesis and characterization of PEGylated, apigenin loaded PLGA nanoparticles**

The experiment starts by synthesizing surface-modified, apigenin-loaded nanoparticles. To enhance the delivery efficiency of nanoparticles, the particles are surface modified with PEG, with the chemistry for surface modifying surface properties of nanoparticles illustrated in Figure 1A. The whole synthesis process starts with producing avidin-palmitate by reacting palmitic acid-NHS with avidin, (Figure 1A up part) followed by producing nanoparticles with avidin palmitate surface. The particles were PEGylated by reacting the avidin group on the nanoparticles and the biotin group in biotin-PEG. (Figure 1A bottom part)Nanoparticle sizes are assessed by dynamic light scattering. The unmodified nanoparticles have a diameter of 141 ± 17 nm and the
size increases to 163± 23 nm after surface modification. (Figure 1B) The unmodified and PEGylated nanoparticles are imaged under SEM, both of which are spherical in shape. (Figure 1C and 1D) Drug loading test shows that the nanoparticles have 9.6% apigenin (96 µg apigenin per 1mg nanoparticles). The release profile of apigenin from nanoparticles is assessed by incubating nanoparticles in PBS buffer. The absorbance at 326 nm is employed as a reference for assessing apigenin concentration. Around 65% of the apigenin is released from the nanoparticles in the first 45 h of incubation, where the rest of apigenin is released continuously in the rest of time. (Figure 1E)

**Dosage and time dependent inhibition of PSC growth by apigenin loaded nanoparticles.**

We next investigate the inhibitory effect of apigenin loaded nanoparticles on PSCs proliferation. Briefly, PSCs at a density of 4 x 10^5 in 6 well plates are treated with apigenin loaded (35 µM) or empty nanoparticles. To assess the effect of treatment time, nanoparticles loaded with certain amount of apigenin are added to cells cultured in culture plates, the number of cells are assessed on different time points. Compared to cells treated with empty nanoparticles, cells treated with apigenin loaded nanoparticles have a significant inhibition on PSC growth over time. (Figure 2A) In the first 36 hours of incubation, cells treated with empty nanoparticles have almost twice as many cells as those treated with apigenin loaded nanoparticles. While PSCs treated with empty nanoparticles proliferate continuously during the treatment, those treated with apigenin loaded nanoparticles do not start to significantly proliferate until 72 hours of treatment. (Figure 2A) We also study the sensitivity of PSCs to apigenin concentration by treating PSCs with different dose of apigenin loaded in nanoparticles. To assess viability, cells are stained with trypan blue, with viability of cells assessed by a cell counter. Our study finds that PSC viability is not significantly affected when apigenin concentration is lower than 10 µM. There is a significant reduce in cell viability when apigenin concentration (loaded in nanoparticles) is increased to 25 µM. (Figure 2B)

**Induced apoptosis of PSCs by apigenin loaded nanoparticles**

Previous study finds that PSCs are highly metabolic active, e.g. limited cell death and high proliferating,\(^9\) which enhance the fibrotic response in injured pancreas. We therefore analyze the apoptosis of PSCs treated with apigenin loaded nanoparticles. Briefly, the cells are treated with apigenin loaded nanoparticles (35 µM
apigenin), with cell apoptosis evaluated over a certain time period. There is no significant cell death in the first 12 h of treatment; the apoptosis rate is significantly increased when incubation time is increased to 48 h. (Figure 3A) We next investigate the efficacy of apigenin/nanoparticles dose on cell viability. Our study finds that apigenin loaded nanoparticles induce PSC apoptosis in a dose dependent manner. (Figure 3B) we do not observe significant apoptosis of PSCs when the apigenin (loaded in nanoparticles) is lower than 20 µM. However, when the dose is increased to 20 to 50 µM, there is significant apoptosis of PSCs, indicating an effective apigenin dose for in vitro treatment of PSCs in this dose range. (Figure 3B)

**Reduced parathyroid hormone-related protein (PTHrP)-associated pro-inflammation and pro-fibrosis mRNA synthesis in PSCs treated with apigenin/nanoparticles.**

PTHrP is a protein that plays a significant role in promoting the fibrosis and inflammation of pancreases. It is usually expressed in a very low level in acinar cells and PSCs within the exocrine pancreas, with no functions reported in basal conditions. As to pancreas with chronic inflammation, previous studies have confirmed the pro-fibrogenic and pro-inflammatory function of PTHrP in pancreatitis. We therefore stimulate PSCs with PTHrP as reported in literature, and assess the role of apigenin loaded nanoparticles in regulating PTHrP-related ECM synthesis. To perform this study, we first stimulate PSCs with 0.5 µM PTHrP for 16 h. The cells are then treated with apigenin loaded nanoparticles (35 µM) for 24 h or PBS (control). After the treatment, RT-PCR is used to investigate the expression of ECM proteins collagen, fibronectin, IL-6 and IL-8 mRNAs which are mRNAs that associate with inflammation and fibrosis in pancreas. Our study finds that an enhanced expression of fibronectin mRNA in PSCs treated with PTHrP, where the expression is reduced to a normal level when cells are treated with both PTHrP and apigenin/NP. We observe the reduction of collagen 1A1 mRNA in PSCs (Figure 4A) Similar trend is also found in another pro-fibrosis associated mRNA, fibronectin mRNA (Figure 4B). As to cytokines associated with pro-inflammation, we assess the expression of IL-6 and IL-8 mRNA in PSCs with different treatments. Our study finds a significant reduction of pro-inflammatory cytokines mRNA (e.g. IL-6 and IL8 mRNAs) in cells treated with both PTHrP and apigenin/NP, as compared to those treated with PTHrP only. (Figure 4C and 4D) We also compare the treatment efficiency of using apigenin loaded in nanoparticles (apigenin/np) Vs. apigenin. Our study finds that while apigenin can slightly reduce the
production of pro-inflammatory and pro-fibrotic mRNAs as compared to control group, it is less efficient than the apigenin loaded in nanoparticles, (Figure 4A-4B) indicating the advantages of using apigenin/np.

**Apigenin loaded nanoparticles reduced stromal fibrosis in mice recurrent acute pancreatitis (RAP) model**

To investigate the *in vivo* efficacy of the apigenin loaded nanoparticles in reducing inflammation and fibrosis of CP, RAP mice model is employed. This model is characterized by a similar pathophysiological features to human CP.\(^{27, 28}\) Briefly, mice are injected hourly with supraoptimal dose of Cerulein, which is a cholecystokinin 1 receptor agonist that can induce acute pancreatitis and then develop CP by the repeated injury in pancreas.\(^{29, 30}\) The RAP model is established 7 days before treatment. Apigenin loaded nanoparticles (144 µg apigenin) is injected into mice daily for 3 weeks of RAP. The interstitial space in the CP tissue is amplified and by inflammatory infiltrate and stromal fibrosis, as stained by fibronectin immunohistochemistry in light color. (Figure 5A) To assess the in vivo circulation of the apigenin nanoparticles, we investigate the concentration of apigenin in mice blood serum. Our study finds that the free apigenin has a slightly higher concentration in serum in the first hour after drug administration. However, there is significant increase of apigenin in serum from hour 24 to hour 72 for mice administrated with apigenin/NP. (Figure 5B) We next dissect pancreases from mice with different treatment, and isolate PSCs from mice with different treatments. RT-PCR is employed to analyze the production of pro-inflammation and fibrosis associated mRNA. Our study finds a slight decrease in the production of pro-inflammatory collagen 1A1mRNA, as compared to mice with no treatment, or mice treated with soluble apigenin. (Figure 5C) We also observe a slight decrease in the production of fibronectin mRNA in PSCs from mice treated with apigenin/NP. (Figure 5D) As to mRNA associated with pancreas pro-inflammation, our study finds a significant decrease in the production of IL-6 and IL-8 mRNAs, (Figure 5E and 5F) indicating the reduction of inflammation in pancreas of these mice.

**DISCUSSIONS**

CP is a disease that imposes strong negative effect on people’s quality of life by associating with patient with uncomfortable symptoms ranging from chronic abdominal pain, diabetes, malnutrition, to impaired digestion, anorexia, even developing pancreatic cancer\(^{2}\) Despite these negative sufferings, no cure exists for CP.
It is therefore necessary to develop new strategies for treating CP. CP is featured by the replacement of parenchymal by scar that results from acute pancreases injury, where the repeated the recurrences of injury at these scars promote the inflammation and fibrosis of CP. Pancreatic stellate cells (PSCs) are the major cells in chronic pancreatitis scaring. PSCs become a major cell for studying CP because the activated PSCs can migrate to injured pancreas, and promote the inflammation and fibrosis at the injury sites. We therefore select this cell as a model for our study.

Recent studies found apigenin can inhibit CP development. However, similar to most therapeutic agents, an effective delivery usually requires a biodegradable carrier to improve its solubility and bioavailability. We therefore used PLGA, a state-of-art material for drug delivery, to load the drug. However, it worth mentioning that, despite finding the recognizing the significant role of PSCs in CP, another challenge facing CP treatment is the lack of effective molecular target for these cells. To enhance the drug delivery efficiency, the particles are therefore modified by PEG via biotin-avidin reactions. (Figure 1A) Surface modifications of nanoparticles are key step in the use of nanoparticles for drug delivery since it improves particle-cell interactions, bio-availability and in vivo circulation time of nanoparticles. We investigate the effect of dose and treatment time of apigenin on PSC viability and apoptosis, finding a dose dependent effect of PSCs growth and apoptosis on apigenin loaded nanoparticles. (Figure 2 and Figure 3) For the time dependent effect, it is probably because the release of PLGA requires certain amount time. (Figure 1D) These results also demonstrate the advantages of using a biodegradable carrier for delivering apigenin, where a sustained delivery of therapeutic agents can be achieved. (Figure 1D) In addition, to improve bio-availability of nanoparticles, we surface modified the nanoparticles with PEG. Our bio-distribution studies show that the nanoparticles have a higher distribution in mice blood over 48 hour, demonstrating the improved delivery efficiency. (Figure 5B)

To further investigate the molecular mechanism of apigenin loaded nanoparticles in treating CP, we stimulate PSCs with PTHrP. (Figure 4) Previous studies find PTHrP can stimulate the expression of pro-fibrosis and pro-inflammation mRNA in PSCs. Consistent with these studies, our study also illustrates the enhanced expression of pro-fibrosis (collagen 1A1 and fibronectin) mRNAs and pro-inflammation mRNAs (IL-6 and IL-8) in PSCs stimulated with PTHrP. (Figure 4A-4D) As to treatment with apigenin loaded
nanoparticles, our study finds a reduction of the pro-fibrosis and pro-inflammation mRNAs, indicating the anti-inflammation and anti-fibrosis of apigenin loaded nanoparticles in CP treatment. (Figure 4A-4D) These studies indicate that the use of apigenin loaded nanoparticles can potential reduce CP development via regulating the inflammatory and fibrotic response. To assess the in vivo efficacy, we inject the apigenin/nanoparticles into mice via tail vein. Our studies find reduction of pro-fibrosis mRNA (collagen 1A1 and fibronectin, Figure 5C and 5D) and pro-inflammation (IL-6 and IL-8, Figure 5E and 5F) in PSCs from mice with CP. Studies by other group find that oral administration of apigenin has anti-fibrotic effect.39,13 While our study confirms these effects, of note is that our study finds that apigenin loaded in PLGA nanoparticles have a longer in vivo circulation time, and stronger anti-inflammatory and anti-fibrosis effect as compared to apigenin administrated in free form. (Figure 5B to 5F)

CONCLUSION

To sum up, this work studies the use of PEGylated, apigenin-loaded PLGA nanoparticles in CP treatment. To further address the mechanism of apigenin in treating CP, our in vitro studies find that apigenin loaded nanoparticles functions as anti-fibrosis agent by inhibiting PSCs proliferation, as well as inducing the PSCs apoptosis. Our study demonstrates that apigenin can inhibit the inflammation and fibrosis in PSCs stimulated with PThrP, which is a mediator that promotes fibrosis and inflammation in CP. In vivo study finds that apigenin loaded nanoparticles inhibit stromal fibrosis and reduce the ratio of fibronectin in mice with CP. This study may provide a potential strategy for the translational use of apigenin for CP treatment.

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Conflict of interest
The authors declare no conflict of interest.

**Ethical standard**

All studied that involves animals were performed under the regulation rules of ethical standards of the institutional and/or national research committee.
REFERENCE


Figure 1. Characterizations of PEGylated, apigenin-loaded PLGA nanoparticles. A) Schematic representation of synthesizing surface modified PLGA nanoparticles. It starts with generating avidin-palmitate by reacting palmitic acid-NHS with avidin, followed by producing nanoparticles with avidin palmitate surface. The particles were PEGylated by reacting the avidin group on the nanoparticles and the biotin group in biotin-PEG. B) Assessment size of surface unmodified nanoparticles (dash line) and PEGlated PLGA nanoparticles (solid line) with dynamic light scattering. Size of nanoparticles were measured by dispersing the particles in DI water and analyzed the intensity distribution of particle sizes. Molecular structure of apigenin is shown in figure 1A (up right). SEM characterization of C) surface unmodified nanoparticles and D) PEGlated PLGA nanoparticles. D) Release profile of apigenin from PEGlated nanoparticles in PBS. Over 70% of apigenin is released in the first 50 hours of incubation.
Figure 2 Effect of apigenin loaded nanoparticles on PSC cell proliferation and viability. A) Proliferation of PSC cells over time. The cells were treated with empty nanoparticles (square label) or apigenin loaded nanoparticles (circular label). Cells treated with apigenin loaded nanoparticles have an inhibited proliferation. B) Viability of PSC treated with different dose of apigenin loaded in nanoparticles. The cells were treated with apigenin loaded in nanoparticles for 72 hour and measured with viability. The cells have a significantly reduced viability when the dose is over 25 µM.
Figure 3. Effect of treatment time and dose on PSC apoptosis. A) Effect of treatment time (12 to 96 h) on PSC apoptosis. The cells were treated with 50 µM apigenin loaded in nanoparticles and measured with apoptosis assay on different time point. B) PSC apoptosis is enhanced by the increasing dose of apigenin/nanoparticles. The cells were treated for 48 h, followed by assessing the apoptosis.
Figure. 4 Apigenin loaded nanoparticles reduce PTHrP induced PSC proliferation, inflammation. The PSC were treated with PTHrP (0.5 µM) for 16 hours, and then stimulated with apigenin loaded in nanoparticles (35 µM) or other control samples. Total RNA of the cells were isolated for reverse transcription-polymerase chain reaction to determine mRNA expression. Expression of A) collagen 1A1, B) fibronectin, C) IL-6, and D) IL-8 mRNAs in PSCs with different treatment. Fold of change is calculated relatively by using the expression of mRNAs in cells treated with empty nanoparticles as a control.
Figure 5. *In vivo* assessment of anti-inflammation and anti-fibrosis effect of apigenin/NP in CP. A) Immunohistochemical staining pancreas with CP. Total RNA of the cells was isolated mice pancreas and assessed with reverse transcription-polymerase chain reaction (RT-PCR) to determine mRNA expression. B) Concentration of apigenin in mice blood after different treatment time. The mice are either treated with soluble apigenin or apigenin loaded in nanoparticles (note as Apigenin/NP in the figures). Mice were sacrificed at different time point with blood obtained to assess the concentration of apigenin in blood. Expression of C) collagen 1A1, D) fibronectin, E) IL-6, and F) IL-8 mRNAs in PSCs from mice with different treatment. Fold of change is calculated relatively by using the expression of mRNAs in the cells from mice treated with empty NP as a standard.