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Nicotine facilitates pancreatic fibrosis by promoting activation of pancreatic stellate cells via α7nAChR-mediated JAK2/STAT3 signaling pathway in rats

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HIGHLIGHTS

- Nicotine facilitates pancreatic fibrosis in CP rat model.
- Nicotine promotes the activation of rPSCs in vivo and in vitro.
- The effect of nicotine is through α7nAChR-mediated JAK2/STAT3 signaling pathway.

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ABSTRACT

Aim: Smoking has been considered as a risk factor of chronic pancreatitis (CP), but the potential mechanism is still unknown. The major pathological feature of CP is pancreatic fibrosis, whose major functional cells are pancreatic stellate cells (PSCs). Nicotine is the major component of cigarette smoke, our recent study suggested that nicotine has the potential to facilitate pancreatic fibrosis in CP. This study was aimed to analyze the function and mechanism of nicotine on PSCs and pancreatic fibrosis in rats.

Materials and methods: In vivo, a rat CP model was induced by intraperitoneal injection of 20 % L-arginine hydrochloride (200 mg/100 g) at 1 h intervals twice per week, nicotine was injected subcutaneously at a dose of 1 mg/kg body weight per day. After four weeks, the pancreatic tissue was collected for H&E, Masson and immunohistochemical staining. In vitro, primary rPSCs were isolated from rats and treated with nicotine (0.1 μM and 1 μM). The proliferation、apoptosis、α-SMA expression、extracellular matrix (ECM) metabolism and α7nAChR-mediated JAK2/STAT3 signaling pathway of rPSCs were detected by CCK-8 assay、flow cytometry、real-time Q-PCR and western blotting analysis. The α7nAChR antagonist α-bungarotoxin (α-BTX) was used to perform inhibition experiments.

Key findings: Nicotine increased pancreatic damage, collagen deposition and activation of PSCs in the CP rat model. In rPSCs, the proliferation, α-SMA expression and ECM formation were significantly promoted by nicotine in a dose-dependent manner. Meanwhile, the apoptosis of rPSCs was significantly reduced after nicotine treatment. Moreover, nicotine also activated the α7nAChR-mediated JAK2/STAT3 signaling pathway in rPSCs. These effects of nicotine on rPSCs were blocked by α-BTX.

Significance: Our finding in this research suggests that nicotine facilitates pancreatic fibrosis by promoting activation of pancreatic stellate cells via α7nAChR-mediated JAK2/STAT3 signaling pathway in rats, partly revealing the mechanism of smoking on chronic pancreatitis.

1. Introduction

Chronic pancreatitis (CP) is a multifactorial, fibroinflammatory syndrome with chronic pain, exocrine and endocrine pancreatic insufficiency, reduced quality of life, and a shorter life expectancy (Beyer et al., 2020). It often originates from irreversible pancreatic tissue damage caused by pancreatic inflammation of variable

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intensity and length (Kleeff et al., 2017). The incidence and prevalence of CP is rising, however, no curative treatment is available.

Pancreatic fibrosis is the major pathological feature of CP (Singh et al., 2019). The major functional cells in the development of pancreatic fibrosis are pancreatic stellate cells (PSCs), which are quiescent and regulate extracellular matrix (ECM) production in normal pancreas. However, the quiescent PSCs can be activated and transformed into myofibroblast-like cells through different signaling pathways when stimulated by various pathogenic factors (Jin et al., 2020; Xue et al., 2018).

Tobacco smoking has been considered as a risk factor of CP, approximately 46 % of cases of pancreatitis were attributable to smoking, and the risks of CP for current smokers were approximately 18 %, the risk of CP was significantly positively correlated with the dose and time of smoking (Tolstrup et al., 2009; Ahmed et al., 2016). But the potential mechanism between smoking and CP is still unknown. Nicotine, the major component of cigarette smoke, has been reported to play a vital role in pancreatic diseases (Ben et al., 2020; Bhattacharjee et al., 2016). In our recent study, we found that nicotine can promote activation of human pancreatic stellate cells (hPSCs), and the α7nAChR-mediated JAK2/STAT3 signaling pathway participated in this process (Li et al., 2020). These findings suggest that nicotine has the potential to facilitate pancreatic fibrosis in CP.

In the current study, we utilized rats for establishing CP models, and assessed the effect of nicotine on pancreatic fibrosis and activation of PSCs. In addition, the isolated primary rat pancreatic stellate cells (rPSCs) were used to analyze the function and mechanism of nicotine on PSC phenotype (proliferation, apoptosis, and collagen synthesis) in this research.

2. Materials and methods

2.1. Animals

Healthy specific pathogen-free male Wistar rats weighing 180–200 g and aged 4–6 weeks were purchased from Beijing Vital River Laboratory Animal Technology. The rats were housed under a 12-h light/dark cycle at 22 ℃. All animal experiments complied with the ARRIVE guidelines and were conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. All methods and procedures were also approved by Ethical Committee of Beijing Chao-yang Hospital, Capital Medical University.

2.2. CP rat models establishment and treatment

Wistar rats were randomized into three groups with 5 rats for each group, control group: normal rats treated with NS; CP group: CP rats treated with NS; nicotine group: CP rats treated with nicotine. Normal rats received NS by intraperitoneal injection at 1 h intervals twice per week. CP rats received 20 % L-arginine hydrochloride (200 mg/100 g) by intraperitoneal injection at 1 h intervals twice per week. The rats of nicotine group were injected subcutaneously with 1 mg/kg body weight nicotine daily, while the control and CP groups were injected with equal volume of NS. The dose selections of L-arginine hydrochloride (Sigma) and nicotine (Sigma) were determined by our pre-experiments. Four weeks later, animals were sacrificed after pancreatic tissues collection. The process of CP model establishment and nicotine treatment was showed in Fig. 1.

2.3. Histological examinations

The fixed pancreatic tissue samples were embedded in paraffin and sectioned at 3 μm. For histological analyses, tissue sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (Solarbio Life Sciences). The histological changes were scored by pathologists, and the fibrotic areas were quantified by Image J software. The percentage of fibrotic area was calculated from the ratio of fibrotic tissue to total pancreatic tissue.

2.4. Immunohistochemical staining

The paraffin-embedded pancreatic tissue sections were immunostained with anti-α-SMA (catalog #ab5694, Abcam), anti-Collagen I (catalog #66761-1-lg, Proteintech), and anti-Collagen III (catalog #ab7778, Abcam). The experiments were performed in accordance with the manufacturer's protocol, images were observed by a microscope (Olympus) and the positive areas were analyzed by Image J software.

2.5. Isolation and culture of rPSCs

Primary rPSCs were isolated from Wistar rats according to a described method used in our previous study (Zhang et al., 2018). The rPSCs were cultured in Dulbecco's modified Eagle medium (DMEM)/Ham's F12 medium (1:1 mixture, Gibco) containing 15 % fetal bovine serum (FBS, Gibco) and 1% Pen/Strep (Gibco). Nicotine (Sigma) or α-bungarotoxin (α-BTX, Sigma) was used to treat rPSCs after cultured with complete medium for 24 h. All cells were maintained in 37℃ and 5% CO2.

2.6. Cell proliferation assays

A Cell Counting Kit-8 (CCK-8) purchased from Dojindo (Japan) was used to evaluate cell proliferation in accordance with the manual instructions. The relative cell viability was calculated as follows: $(A_{treatment} - A_{blank})/(A_{control} - A_{blank}) \times 100$ %.

2.7. Flow cytometry

A PE Annexin V Apoptosis Detection Kit I purchased from BD Biosciences was used to analyze the apoptosis of rPSCs as described in manufacturer's protocol, and the data was analyzed by FlowJo v10 software.

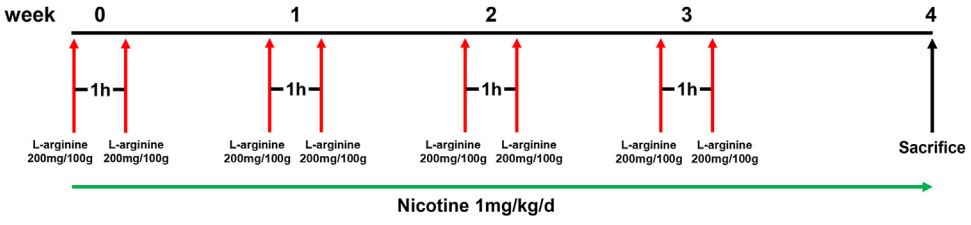

Fig. 1. Process of CP model establishment and nicotine treatment.

Table 1
List of primers used for real-time Q-PCR.

Gene	Primer sequences (5'to3')
GAPDH	F: GGCAAGTTCAACGGCACAG
	R: CGCCAGTAGACTCCACGACAT
α-SMA	F: CACCATCGGGAATGAACGCT
	R: CGAGAGGACGTTGTTAGCATAGAG
COL1	F: GACATGTTCAGCTTTGTGGACCTC
	R: GGGACCCTTAGGCCATTGTGTA
COL3	F: TCCCAGAACATTACATACCACT
	R: GCTATTTCCTTCAGCCTTGA
TIMP1	F: AGCCCTGCTCAGCAAAAGG
	R: CTGTCCACAAGCAATGACTGTCA
MMP13	F: GTGACTCTTGCGGGAATCCT
	R: CAGGCACTCCACATCTTGGT
TIMP2	F: TGCACCCGCAACAGGCGTTTT
	R: TTCCTCCAACGTCCAGCGAGA
MMP2	F: CACCAAGAACTTCCGACTATCCA
	R: ACCAGTGTCAGTATCAGCATCAG

F: forward; R: reverse.

2.8. Real-time quantitative polymerase chain reaction (Q-PCR)

In accordance with the manufacturer's instructions, the total RNA of rPSCs was isolated by Trizol reagent (Invitrogen). 1 μg of total RNA of each sample was subjected to reverse transcription carried with PrimeScrip RT Master Mix (Taraka) guided by the protocol. Q-PCR was performed using an ABI 7500 system with SYBR Green PCR Master Mix (Applied Biosystems) and specific gene primers (Table 1). The GAPDH gene was used as an internal standard to calculate relative mRNA levels with Livak (2-ΔΔCt) method.

2.9. Western blotting analysis

Cells were prepared using RIPA (Solarbio) lysate and total proteins were collected. BCA Protein Assay Kit (Beyotime Biotechnology) was used to measure the concentrations of protein. An equal amount of protein (40 μg) was loaded into SDS-PAGE using 10 % running gels and transferred onto nitrocellulose (NC) or PVDF membranes. The list of primary antibodies was shown in Table 2. The average intensities of protein bands were calculated by Image J software.

2.10. Statistical analysis

The data was expressed as mean ± SD for at least three experiments and analyzed by SPSS 18.0. Two-tailed Student's *t*-test was used to examine differences between 2 groups, one-way ANOVA was performed to contrast intergroup variance among multiple groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Nicotine exacerbates pancreatic fibrosis in CP rat model

HE staining was used to detect the morphological changes of rat pancreatic tissue (Fig. 2A, B). After 4 weeks, the CP group showed obvious histological changes in pancreas compared with the control group. Compared with the CP group, the pancreatic damage was significantly enhanced in rats treated with nicotine. From Masson staining (Fig. 2A, C), CP rats showed obvious collagen accumulation in pancreas. After nicotine treatment, collagen deposition was extremely obvious in pancreas.

3.2. Nicotine aggravates the activation of PSCs and ECM formation in rat pancreas

The high expression level of α-SMA indicated activation of PSCs. As seen in the immunohistochemistry staining, the expression of α-SMA was increased significantly in CP group while the level of α-SMA expression became higher after nicotine treatment (Fig. 3A, B). The ECM components, including Col I and Col III, were also detected by immunohistochemistry staining. The result showed that expression of Col I and Col III were significantly increased in CP rats, while became higher after nicotine treatment (Fig. 3A, C, D).

3.3. Nicotine promotes the proliferation of rPSCs

Different concentrations of nicotine (0.1 μM, 1 μM) were used to treat rPSCs for different times (24 h, 48 h). The CCK-8 assay was used to evaluate the effect of nicotine on the proliferation of rPSCs. The result has showed that nicotine can induce the proliferation of rPSCs in a time and dose-dependent manner (Fig. 4A, B). Because nicotine had a great effect on rPSCs after 48 h treatment, we deepen our studies on rPSCs after 48 h with nicotine treatment. Another proliferation marker cyclin D1 was also showed significantly higher expression in nicotine-treated groups than control group in a dose-dependent manner by western blotting analysis (Fig. 4C, D).

3.4. Nicotine suppresses the apoptosis of rPSCs

The flow cytometry was used to analyze the level of apoptosis in rPSCs. After incubation with 0.1 μM and 1 μM nicotine, the apoptotic rates of rPSCs were showed significantly lower compared to the control group (Fig. 5A, B). The expression of apoptosis-related proteins Bax and Bcl-2 were also tested using western blotting analysis. The result revealed that the ratio of Bax/Bcl-2 was significantly decreased in rPSCs which treated by 0.1 μM and 1 μM nicotine (Fig. 5C, D).

Table 2
List of antibodies used for western blotting analysis.

Antibody	Catalog number	Species	Source
GAPDH	#2118	Rabbit	Cell Signaling Technology
α-SMA	ab5694	Rabbit	Abcam
Collagen I	#84336	Rabbit	Cell Signaling Technology
Collagen III	ab7778	Rabbit	Abcam
Cyclin D1	#55506	Rabbit	Cell Signaling Technology
Bcl-2	#2876	Rabbit	Cell Signaling Technology
Bax	#2772	Rabbit	Cell Signaling Technology
α7nAChR	ab10096	Rabbit	Abcam
Phospho-Jak2	#3776	Rabbit	Cell Signaling Technology
Phospho-Stat3	#9145	Rabbit	Cell Signaling Technology
Jak2	#3230	Rabbit	Cell Signaling Technology
Stat3	#30835	Rabbit	Cell Signaling Technology

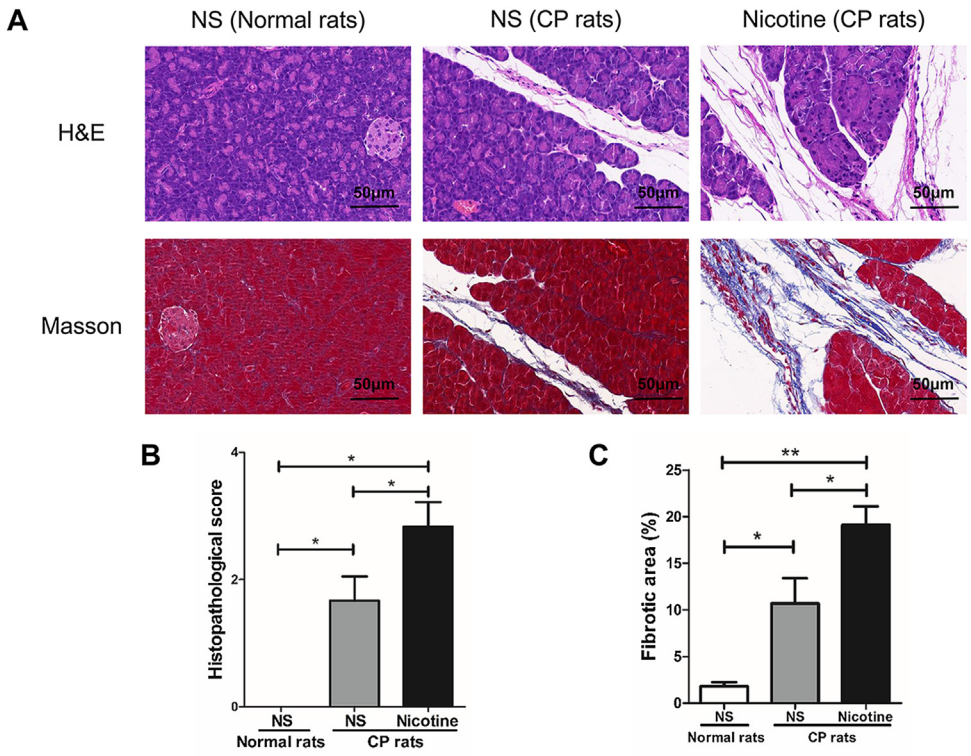

Fig. 2. Nicotine exacerbates pancreatic fibrosis in CP rat model. (A) Pancreatic histological changes were observed by H&E and Masson staining (400×). (B) The histopathological score of H&E staining. (C) Quantitative analysis of fibrotic areas detected by Masson staining. (*, $p < 0.05$, **, $p < 0.01$).

3.5. Nicotine increases the expression of α-SMA in rPSCs

The expression of α-SMA was measured to evaluate the activation of rPSCs by real-time Q-PCR and western blotting analysis. The levels of α-SMA protein were significantly higher in nicotine treated groups compared to the control group as shown in western blotting analysis (Fig. 6A, B). The results of real-time Q-PCR suggested that the mRNA levels of α-SMA were consistent with protein levels (Fig. 6C).

3.6. Nicotine facilitates the ECM formation of rPSCs

The western blotting analysis was used to detect the expression of Col I and Col III protein. Compared to the control group, the

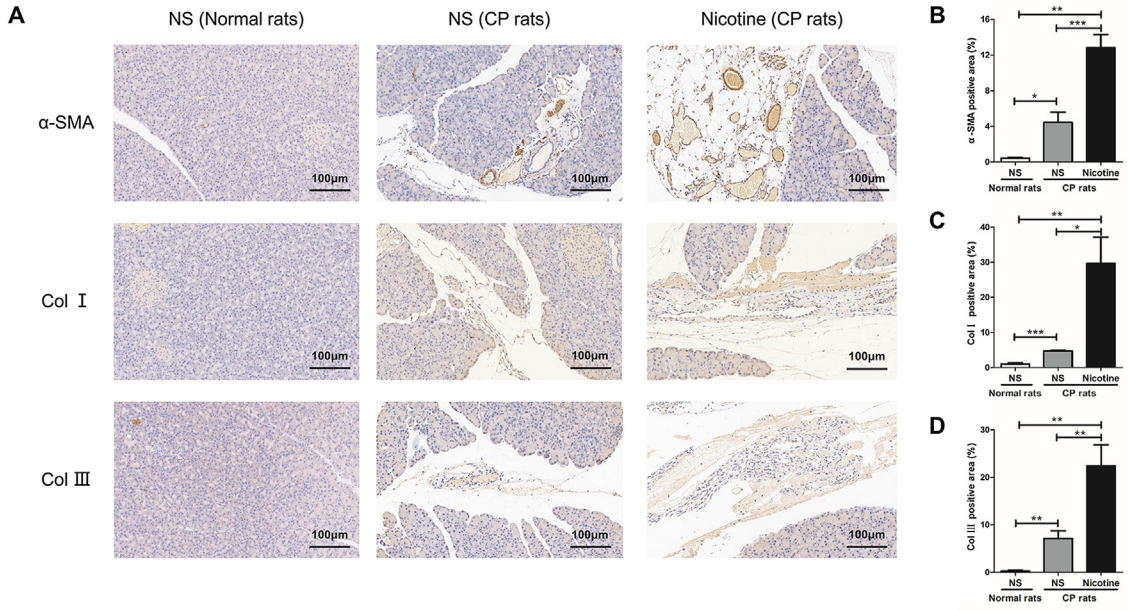

Fig. 3. Nicotine aggravates the activation of PSCs and ECM formation in rat pancreas. (A) Immunohistochemistry staining showing the expression of α-SMA, Col I and Col III in pancreatic tissue (200×). (B) Quantitative analysis of α-SMA positive areas detected by immunohistochemistry staining. (C) Quantitative analysis of Col I positive areas detected by immunohistochemistry staining. (D) Quantitative analysis of Col III positive areas detected by immunohistochemistry staining. (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

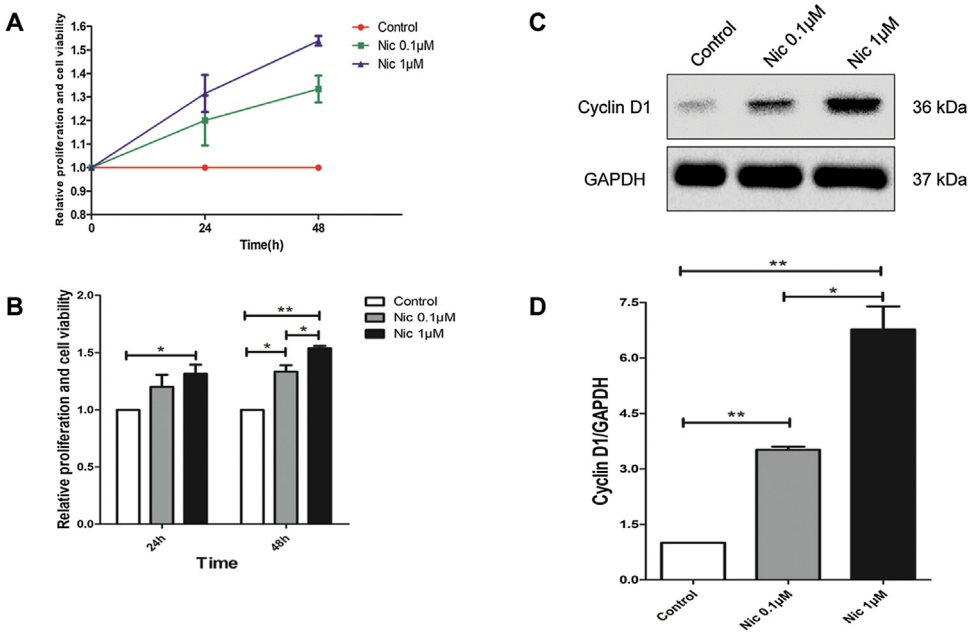

Fig. 4. Nicotine promotes the proliferation of rPSCs. (A, B) Relative proliferation and cell viability of rPSCs treated by nicotine (0, 0.1, 1 μM) for 24 h and 48 h, determined by CCK-8 assays. The data are expressed as fold changes over the control group. (C) Western blotting analysis for Cyclin D1 expression of rPSCs after treatment with nicotine for 48 h. (D) Quantification of western blotting analysis for Cyclin D1 expression of rPSCs after treatment with nicotine for 48 h compared to control group. (*, $p < 0.05$, **, $p < 0.01$).

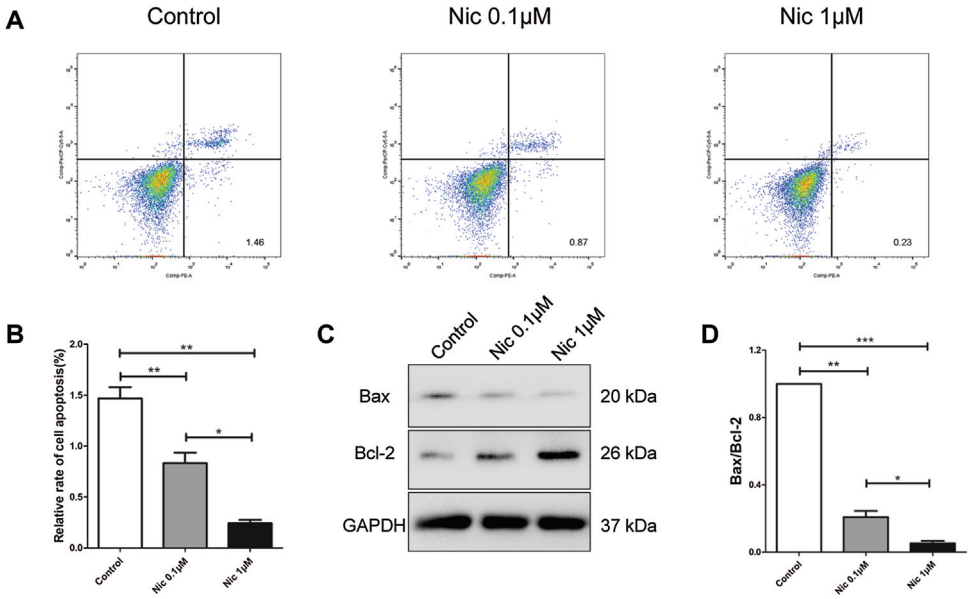

Fig. 5. Nicotine suppresses the apoptosis of rPSCs. (A, B) Apoptotic rates of rPSCs treated by nicotine for 48 h, measured by flow cytometry. (C) Western blotting analysis for Bax and Bcl-2 expression of rPSCs after treatment with nicotine for 48 h. (D) Quantification of western blotting analysis for Bax/Bcl-2 ratio of rPSCs after treatment with nicotine for 48 h compared to control group. (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

nicotine-treated groups showed a significantly higher expression of Col I and Col III protein (Fig. 7A-C). Furthermore, the results of real-time Q-PCR also displayed significantly higher mRNA levels of Col I、Col III、TIMP1 and TIMP2, with significantly lower mRNA levels of MMP13 and MMP2 in nicotine-treated groups compared with the control group (Fig. 7D-I).

3.7. Nicotine activates α7nAChR-mediated JAK2/STAT3 signaling pathway in rPSCs

We had proved the positive effect of nicotine on the activation of rPSCs. In order to explore the molecular mechanism of nicotine-induced rPSCs activation, we studied the α7nAChR-mediated JAK2/

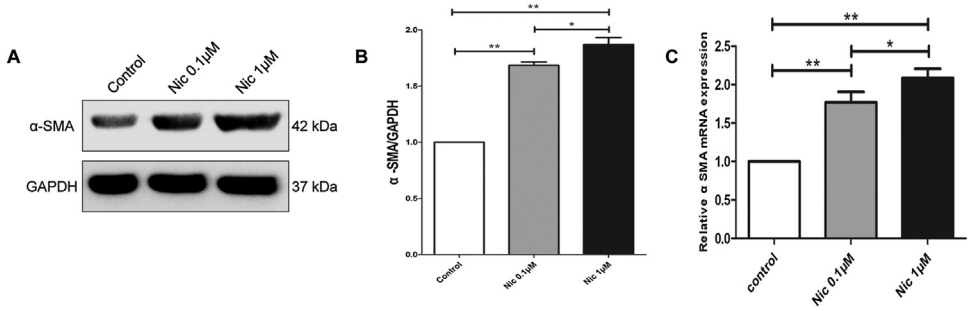

Fig. 6. Nicotine increases the expression of α-SMA in rPSCs. (A) Western blotting analysis for α-SMA expression of rPSCs after treatment with nicotine for 48 h. (B) Quantification of western blotting analysis for α-SMA expression of rPSCs after treatment with nicotine for 48 h compared to control group. (C) Relative α-SMA mRNA level of rPSCs after treatment with nicotine for 48 h, detected by real-time PCR. (*, $p < 0.05$, **, $p < 0.01$).

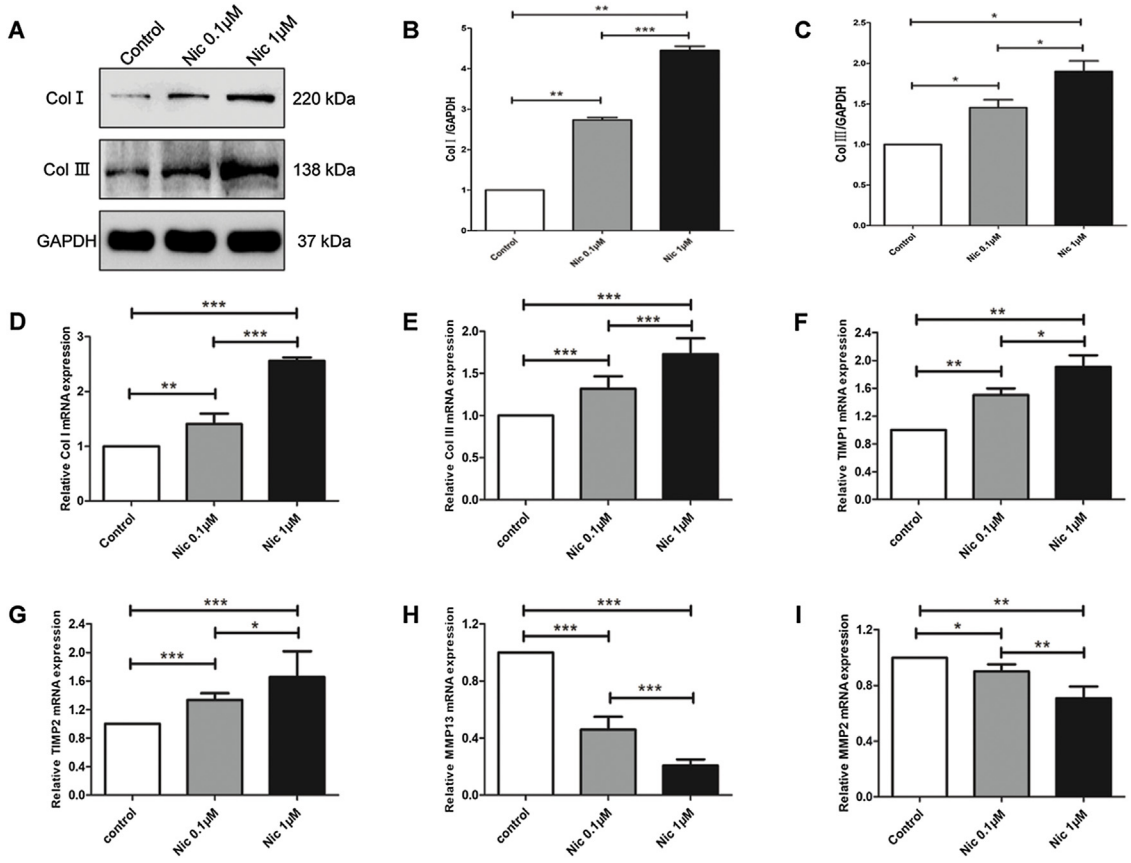

Fig. 7. Nicotine facilitates the ECM formation of rPSCs. (A) Western blotting analysis for Col I and Col III expression of rPSCs after treatment with nicotine for 48 h. (B) Quantification of western blotting analysis for Col I expression of rPSCs after treatment with nicotine for 48 h compared to control group. (C) Quantification of western blotting analysis for Col III expression of rPSCs after treatment with nicotine for 48 h compared to control group. (D-I) Relative mRNA level of Col I、Col III、TIMP1、MMP13、TIMP2、MMP2 in rPSCs after treatment with nicotine for 48 h, detected by real-time PCR. (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

STAT3 signaling pathway by western blotting analysis. As showed in Fig. 8, nicotine significantly increased the expression of α7nAChR、p-JAK2 and p-STAT3 in a dose-dependent manner, suggesting that nicotine can activate α7nAChR-mediated JAK2/STAT3 signaling pathway in rPSCs.

3.8. Nicotine promotes the activation of rPSCs via α7nAChR-mediated JAK2/STAT3 signaling pathway

Because nicotine had the most significant effect on the rPSCs with the concentration of 1 μM, we continued our inhibition experiment with 1 μM nicotine treatment. At the same time, α-BTX, a specific antagonist of α7nAChR, with the concentration of 0.1 μM, was used to block α7nAChR-mediated JAK2/STAT3 signaling pathway 30 min before nicotine treatment. The results of western blotting analysis reflected that the expression of α7nAChR、p-JAK2、p-STAT3、α-SMA was significantly decreased in nicotine combined α-BTX group compared with nicotine group (Fig. 9), implying that nicotine promotes the activation of rPSCs via α7nAChR-mediated JAK2/STAT3 signaling pathway.

4. Discussion

Recently, a few clinical studies revealed that smoking has a close relationship with clinical manifestations and pancreatic function damage of CP (Aslam et al., 2021; Tjora et al., 2020). Our

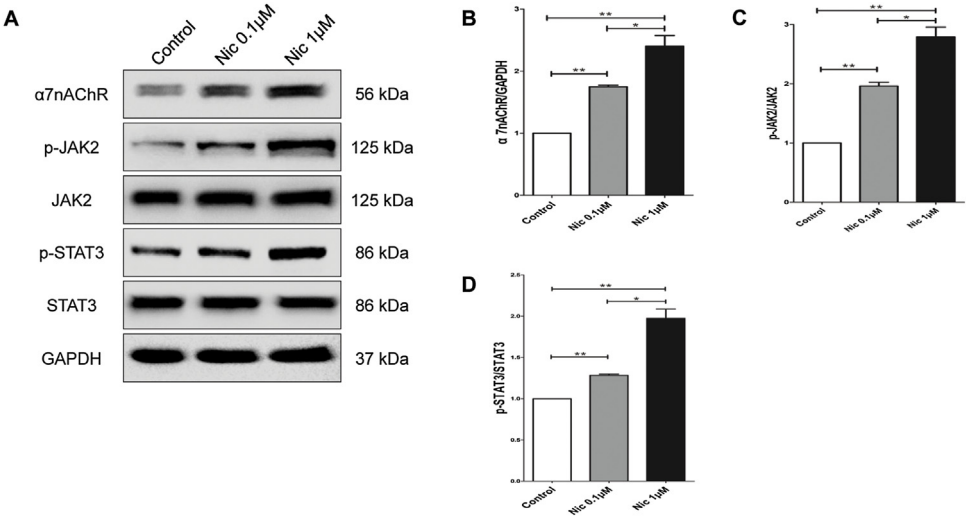

Fig. 8. Nicotine activates α7nAChR-mediated JAK2/STAT3 signaling pathway in rPSCs. (A) Western blotting analysis for α7nAChR、p-JAK2、JAK2、p-STAT3 and STAT3 expression of rPSCs after treatment with nicotine for 48 h. (B) Quantification of western blotting analysis for α7nAChR expression of rPSCs after treatment with nicotine for 48 h compared to control group. (C) Quantification of western blotting analysis for p-JAK2/JAK2 ratio of rPSCs after treatment with nicotine for 48 h compared to control group. (D) Quantification of western blotting analysis for p-STAT3/STAT3 ratio of rPSCs after treatment with nicotine for 48 h compared to control group. (*, $p < 0.05$, **, $p < 0.01$).

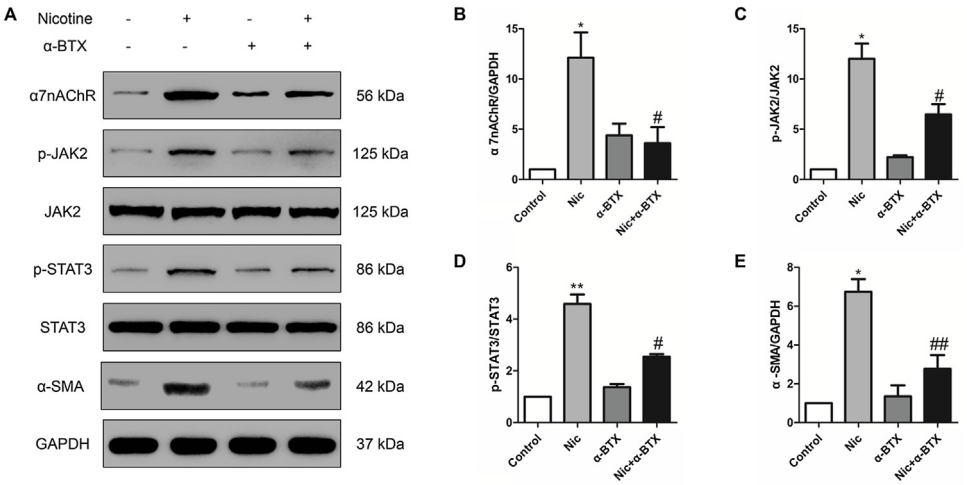

Fig. 9. Nicotine promotes the activation of rPSCs via α7nAChR-mediated JAK2/STAT3 signaling pathway. (A) Western blotting analysis for α7nAChR、p-JAK2、JAK2、p-STAT3、STAT3、α-SMA expression of rPSCs after treatment with nicotine、α-BTX or nicotine combined α-BTX for 48 h. (B) Quantification of western blotting analysis for α7nAChR expression of rPSCs compared to control group. (C) Quantification of western blotting analysis for p-JAK2/JAK2 ratio of rPSCs compared to control group. (D) Quantification of western blotting analysis for p-STAT3/STAT3 ratio of rPSCs compared to control group. (E) Quantification of western blotting analysis for α-SMA expression of rPSCs compared to control group. (*, $p < 0.05$ vs control group, **, $p < 0.01$ vs control group, #, $p < 0.05$ vs nicotine group, ##, $p < 0.01$ vs nicotine group).

early study also provided evidence for cigarette smoke-induced pancreatic damage in rats (Jianyu-Hao et al., 2009). But the mechanism of smoke-induced CP remains unclear. In current study, we confirmed the positive effect of tobacco's major component nicotine on the progression of CP and its related mechanism.

As we all know, pancreatic fibrosis is the main pathological change with CP. In our previous study, we found that nicotine could not cause histological changes in the pancreas of normal rats. Therefore, we guessed that the main effect of nicotine was to aggravate the destruction of pancreas in the presence of CP. In this study, we established a CP animal model by a feasible method we have previously reported, then detected the fibrotic state of pancreas (Xue et al., 2019). At the same time, the CP rats were injected subcutaneously with 1 mg/kg body weight nicotine daily, this was a method that can simulate the concentration in the body of smokers (Russell et al., 1980; McMillan and Tyndale, 2015). The results indicated that our CP models were successful and the use of nicotine can aggravate pancreatic fibrogenesis in CP animals. This function of nicotine was also found in myocardial fibrosis, kidney fibrosis and lung fibrosis (Yu et al., 2016; Chen et al., 2015; Huang et al., 2014).

The formation of pancreatic fibrosis is mainly due to the production and deposition of ECM, which is attributed to the activation of PSCs. The activated PSCs are characterized by enhanced proliferation, suppressed apoptosis, high expression of α-SMA, excessive production of Col I and Col III, decreased MMPs, and increased TIMPs (Zhang et al., 2018; Cui et al., 2020). In our present study, we demonstrated that nicotine significantly increases the degree of the activation of rPSCs whether in vivo or in vitro. These results were consistent with the effect of nicotine on hPSCs in our previous in vitro study (Li et al., 2020). But the

difference was, with respect to hPSCs only react to high-dose nicotine (1 μM), while rPSCs also respond to low-dose nicotine (0.1 μM), although this effect was significantly weaker than high-dose nicotine. Perhaps this difference can be explained by different sensitivity of different species to nicotine.

The nicotinic acetylcholine receptor alpha 7 (α7nAChR) and its downstream JAK2/STAT3 pathway have been found to play important roles in multiple functions of the body (Cao et al., 2020; Zhang et al., 2020). In our previous study, we also found that nicotine promotes the activation of hPSCs through α7nAChR-mediated JAK2/STAT3 signaling pathway (Li et al., 2020). In our current study, we got the same result in rPSCs, providing a stronger evidence for the mechanism of nicotine affecting PSCs.

In this study, we first confirmed the facilitating effect of nicotine on pancreatic fibrosis through an animal model of CP, and revealed inner mechanism together with our previous study, partly elucidating the correlation between smoking and the pathogenesis of CP.

Meanwhile, there are still some limitations in this study. Firstly, nicotine was injected subcutaneously in our experiment, but in smoking situations it will be through lungs or by oral in tobacco chewing scenarios, we will use the method of cigarette smoke inhalation and the method of oral nicotine solution to verify the results of this study in the future. Secondly, our CP models were established by L-arginine, this is just one of several ways for building CP models, we are continuing to use other methods to establish CP models to verify our findings, such as EtOH + LPS, caerulein, and so on. Lastly, we must be soberly aware that the composition of tobacco is very complex, the research we have done is just the tip of the iceberg, to completely study clearly the relationship between smoking and CP, we still have a long way to go.

5. Conclusion

In conclusion, our finding in this research suggests that nicotine facilitates pancreatic fibrosis by promoting activation of pancreatic stellate cells via α7nAChR-mediated JAK2/STAT3 signaling pathway in rats, partly revealing the mechanism of smoking on chronic pancreatitis.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

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