The inhibitory effects of Nardostachys jatamansi on alcoholic chronic pancreatitis

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Nardostachys jatamansi (NJ) belonging to the Valerianaceae family has been used as a remedy for gastrointestinal inflammatory diseases for decades. However, the potential for NJ to ameliorate alcoholic chronic pancreatitis (ACP) is unknown. The aim of this study was to examine the inhibitory effects of NJ on ACP. C57black/6 mice received ethanol injections intraperitoneally for 3 weeks against a background of cerulein-induced acute pancreatitis. During ACP, NJ was ad libitum administrated orally with water. After 3 weeks of treatment, the pancreas was harvested for histological examination. NJ treatment increased the pancreatic acinar cell survival (confirmed by amylase level testing) and reduced collagen deposition and pancreatic stellate cell (PSC) activation. In addition, NJ treatment reduced the activation but not death of PSC. In conclusion, our results suggest that NJ attenuated ACP through the inhibition of PSC activation. [BMB Reports 2012; 45(7): 402-407]

INTRODUCTION

Prominent fibrosis, inflammation, glandular atrophy, and ductal changes are major histological features of chronic pancreatitis (CP) (1). The resulting symptoms of abdominal pain, steatorrhea, and weight loss may be debilitating, complicated by psychosocial problems, loss of work, narcotic addiction, and consumption of health-care resources (2). CP is mainly associated with alcohol abuse (3). The majority of patients are male with an average alcohol consumption of 150 g per day for 10-15 years from the initial date of presentation, since alcoholic acute pancreatitis (AP) rarely occurs after a single binge.

Abnormal deposits of fibrous tissue are characteristic histological features of two major diseases of the pancreas such as CP, and pancreatic cancer (4). The key players of chronic injury of pancreas are pancreatic stellate cells (PSCs) (5). The ability of culture PSCs provided a much needed in vitro tool enabling researchers to examine PSC biology in health as well as in disease states. The deactivation or extermination the PSCs is well-known treatment of CP (6).

We already have reported that Nardostachys jatamansi (NJ) has protective effects on acute pancreatitis, and sepsis (7, 8). We have used the root decoction in the evaluation of NJ’s effects. NJ has been used in mental disorders, insomnia, blood disorders, and the circulatory system (9). Various sesquiterpenes such as jatamansic acid and jatamansone, lignans, and neolignans are present in the roots of plants (10, 11). Although many studies have approached the anti-inflammatory effects, the anti-fibrotic effect of NJ on ACP has not been examined.

In the present study, we evaluated the effect of NJ on ACP. To reach the goal, we used the repetitive AP plus alcohol model. We also examined the morphological and histological changes in the pancreas, and measured the collagen deposition and PSC activations in vivo and in vitro.

RESULTS

Effect of NJ on morphological and histological examination of pancreas during ACP

We investigated the morphological and histological architecture of pancreas against ACP. In our study, following a 3 week challenge, pancreatic interstitial edema, inflammatory, infiltration, and pancreatic fibrosis were evident (Fig. 1). The ACP lost the typical morphology, and resulted in a more edematous than normal pancreas. Treatment of 5 or 10 mg/ml of NJ reduced the pancreatic outer morphological edema significantly (Fig. 1). However, on histological examination, the NJ treatment did not show the dramatic protection against ACP challenge (Fig. 1B). To further examine the exact effect of NJ on ACP, we evaluated the pancreatic acini survival, which secretes amylase. ACP challenged mice demonstrated a destruction of pancreatic acini, as is shown by reduced amylase. However, NJ treatment dramatically inhibits the acinar cell death and destruction (Fig. 2).
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The anti-fibrotic effect of *Nardostachys jatamansi* was studied in the context of pancreatic fibrosis. The study involved the use of the plant's extract (NJ) in a model of acute pancreatitis (ACP) to assess its effects on fibrosis.

**Effect of NJ on fibrosis**

When PSCs are stimulated and activated, α-SMA expression, collagen deposition, and cytokine production are increased (12). Therefore, in vivo PSC activation was assessed in the model by immunostaining the pancreas with the α-SMA, the activation marker during ACP. However, NJ treated ACP mice had shown a decreased α-SMA expression in the pancreas (Fig. 3A). Measurement by real-time RT-PCR of mRNA levels of the Acta2, Fn1, Tgfβ genes indicated that the levels of gene expression of α-SMA, fibronectin1, and tumor growth factor (TGF)-β had increased significantly in ACP-challenged mice. The increase of mRNA expression was reduced by NJ treatment during ACP (Fig. 3B). Next, to determine the effect of NJ on fibrosis during ACP, we conducted a histological evaluation of pancreatic collagen deposition. ACP caused a marked increase in collagen expression in the pancreas. However, collagen production was significantly reduced in NJ treated mice (Fig. 3C).

**Effect of NJ on PSC activation**

To specify the role of NJ on ACP, we isolated the PSC from the rat pancreas. PSCs express intermediate filament proteins such as desmin (13). As previously reported, isolated PSC had a desmin positive response, which means that isolated cells are PSC (Fig. 4A). At the 3 week culture of PSC, the PSCs are activated and ex-
press α-SMA (Fig. 4B). However, NJ treatment inhibited the expression of α-SMA in PSC (Fig. 4B and C). NJ treatment also inhibited the mRNA expression of fibrosis-related genes such as α-SMA, fibronectin1, and TGF-β in PSC.

**DISCUSSION**

Fibrosis of the pancreas has long been considered as an end stage with little or no hope for improvement. It is only with the better understanding of the physiological mechanisms underlying CP and characterization of the stellate cell as the main effector cell in this process that the potential for fibrosis to regress has received more attention. In this study, using a recently reported model of ACP (14), we demonstrated that NJ reduced the development of ACP, as shown by an inhibition of pancreatic acinar cell destruction, collagen deposition and PSC activation.

AP is characterized by pain, edema, hemorrhage, acinar cell vacuolization, necrosis, inflammation, and increased serum amylase and lipase; these events are confounded in the chronic form of the disease by fibrosis, inflammation, collagen deposition and decreased exocrine and endocrine function. Although AP and CP differ in epidemiology, repeated AP can gradually result in the development of CP (15). However, the repetitive administration of the cerulein model has been limited for treatment time, severity, and human similarity. Therefore, we added alcohol consumption to the repetitive AP model to induce ACP because chronic excessive alcohol consumption is a major risk factor for developing chronic pancreatitis (16-19). The 3 week challenge given to mice was sufficient to induce ACP, which was proven by fibrosis, inflammation, and acinar cell destruction in the pancreas (Fig. 1). Daily and free consumption of NJ inhibited the morphological damage against ACP (Fig. 1). Although the histological protection is not significant, amylase positive cells were abundant in the pancreas, suggesting that the exocrine functions may have been working properly.

For a repetitive AP, the pancreas encountered inflammation and necrosis, which ultimately resulted in the release of cytokines and other stimulatory factors (20-22). When activated by pro-fibrogenic mediators such as TGF-β, PSCs transform into myofibroblast like cells expressing α-SMA, and fibronectin and collagens are deposited near the fibrotic area (23). In this experi-
ment, we detected a significant increase of collagen deposits, α-SMA, fibronectin and TGF-β in ACP mice. Also activated PSCs (confirmed by desmin) had shown an increased α-SMA, fibronectin and TGF-β. However, the fibrotic response was significantly attenuated by NJ treatment, as shown by a reduction of α-SMA, fibronectin, TGF-β and collagen deposits. These results suggest that reduced fibrosis might be involved in PSC deactivation through a TGF-β production inhibition.

The major question in the patho-physiology of ACP is whether the disease status can be reversed. Many clinicians routinely advise patients to abstain from alcohol, however this has no benefit for the patient. Previous studies that have tried to reverse ACP by stopping alcohol consumption, they could not find a significant improvement (2, 24). In contrast to humans, the murine AP and ACP model generally recover the diseases after the absence of stimuli (25, 26). Thus, the murine experimental model is not enough to prove the mechanisms of human CP. However, we have to focus the recovering mechanisms of the experimental model to provide a clinical source to treat human CP. In the regression of ACP, the regulation of PSC is a key factor (12). Indeed, many studies tried to regulate PSC by deactivating or killing the PSC factor (27-32). In our ACP model, NJ treatment resulted in the deactivation of PSCs, as shown by a reduction of α-SMA, fibronectin, and collagen expressions in pancreas. Prior to our report (7), NJ treatment attenuated the severity of cerulein-induced pancreatitis. Therefore, we want to rule out the possibility that NJ reduced severity of repeated pancreatitis. Using isolated rat PSC, the direct effect of NJ on PSC activation was examined. NJ treatment did not induce PSC death (data not shown). As shown in Fig. 4, NJ reversed the fully activated PSC, as shown by reduced fibrotic marker. These results suggest that the protective mechanisms of NJ against fibrosis may be deactivating PSCs but not killing PSC.

We have shown that administration of NJ prevented the progression of pancreatic fibrosis in mice. Although the histological protection was not significant, reduction of inflammation and collagen deposition might suggest the possibility of NJ on ACP. In conclusion, our results could support that NJ has an anti-fibrotic effect against ACP through deactivation of PSCs.

MATERIALS AND METHODS

Materials

Cerulein, Tris-HCl, NaCl, collagen, DAPI, hematoxylin, eosin, xylene, ethanol, cerulein and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The α-smooth muscle actin (SMA), collagen, and desmin were purchased from Abcam (UK). Amylase and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals

All experiments were performed according to protocols approved by the Animal Care Committee of Wonkwang University. 6-8-weeks-old C57BL/6 mice weighing 15-20 g were purchased from Orient Bio (Sungnam, KyungKiDo, South Korea). All animals were bred and housed in standard shoebox cages in a climate-controlled room with an ambient temperature of 23 ± 2°C and a 12 hour light-dark cycle for 7 days. Animals were fed a standard laboratory chow, allowed water ad libitum, and randomly assigned to control or experimental groups. The mice were fasted for 18 h before induction of ACP.

Experimental design

We used rapid and efficient model which has been reported from Charrier and Brigstock (14). Briefly, C57Bl/6 mice 6-8 weeks old were injected with ethanol (3.2 g/kg; administered in a 33.3% ethanol: 67.7% water solution) i.p. one time per day, six times per week, for 3 weeks. On one day each week, mice also received an i.p. injection of cerulein every hour for 6 hours (50 μg/kg). Mice were housed three to a cage and fed a low-fat diet ad libitum. Mice were killed 1 day after the last ethanol treatment and the pancreata were removed before fixation in 4% paraformaldehyde (pH 7.2-7.4) or for RNA and protein extraction.

Histology

Fixed pancreatic tissues were embedded in paraffin, cut into 4 μm sections and stained with haematoxylin-eosin for standard histological examination. Immunohistochemical staining for amyrase, collagen, or α-SMA was performed using DAB immunohistochemical kit (DAKO, Cytoration, Denmark), Immunofluorescent detection of DAPI, α-SMA or desmin was achieved by incubation of PSC with anti-α-SMA, or desmin followed by incubation with Alexa Fluors 568 goat-anti rabbit IgG (1 : 2,000, Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. Slides were mounted with mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA).

Messenger RNA (mRNA) expression

Transcriptions of target cytokines were analyzed by RT-PCR in mouse pancreatic tissues and PSCs. Total RNA was isolated from the mouse pancreata and PSCs using Trizol (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription using SuperScript II RT (Invitrogen, Carlsbad, CA, USA). TaqMan quantitative RT-PCR using the ABI stepone plus system was performed according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of the gene of interest and the results were normalized to those of the “housekeeping” hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA. Arbitrary expression units were calculated by the division of expression of the gene of interest by ribosomal protein HPRT mRNA expression. The sequences of forward, reverse, and probe oligonucleotide primers for multiplex real-time TaqMan PCR were purchased from ABI (Invitrogen, Carlsbad, CA, USA).

PSC isolation

Rat PSCs were prepared from the pancreas tissues of male Wistar
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rats (Orient Bio, Sungnam, KyungKiDo, South Korea) weighting 250 to 300 g according to the procedure described by Shinji et al. (33). In brief, the pancreas was digested with 0.03% collagenase P in Hank’s buffered salt solution. The resultant suspension of cells was centrifuged in a 13.2% iohexol gradient at 1,400 g for 20 min. Stellate cells were separated into a fuzzy band just above the interface of the iohexol solution and the aqueous buffer. This band was harvested, and the cells were washed and resuspended in DMEM containing 10% fetal bovine serum (FBS), 4 mM glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml). After reaching confluency, cells were harvested and replated at equal seeding densities. All experiments were performed using culture-activated cells (passages 2-4). PSCs were incubated in a serum-free medium for 24 h before the addition of experimental reagents.

**Western blotting**

PSCs were harvested, then the lysates were boiled in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Following transfer of protein, the membrane was blocked with 5% skim milk in PBS-Tween-20 (PBST) for 2 hours at RT and then incubated with antibodies to α-SMA overnight. After washing 3 times, each blot was incubated with peroxidase-conjugated secondary antibody for 1 hour and the antibody-specific proteins were visualized using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) according to the manufacturer’s recommended protocol.

**Statistical analysis**

Results are expressed as means ± SE. The significance of changes was evaluated using two-way ANOVA, with time and dose parameters. Differences between the experimental groups were evaluated using analysis of variance. Values of P < 0.05 were accepted as statistically significant.

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**REFERENCES**


