Original Article

Poria cocos Herbal Acupuncture Prevents β-cell Damage on Streptozotocin-induced Diabetic Rat

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국문초록

Streptozotocin 유도 당뇨 흰쥐에서 복령약침의 β-cell 손상 방지 효과

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목적 : 정상 췌장조직 속에 존재하는 췌장 소도세포들을 파괴시켜 고혈당을 유발시키고 복령 물추출물로 약침을 시술하여 췌장 조직의 보호효과와 항당뇨 효과를 살펴보고자 실험을 진행하였다.

방법 : 5주령의 Sprague-Dawley rat을 통제된 실험실 환경에 적응시킨 후 1주일간 복령약침액(125mg/kg 복령약침군 및 250mg/kg 복령약침군)을 좌우 신수(BL_{23})에 교대로 각각 피하에 약침하고 streptozotocin을 복강내 주사하여 3일 후 diabetes mellitus 유도 정도를 평가하고 2주일간 추가 치료를 진행한 뒤, 혈액지표(plasma glucose, insulin, TG, TC, NEFA, sGOT, sGPT, ALP, BUN, CRE)와 췌장조직의 형태학적 분석 및 염증 관련 단백질의 발현을 평가하였다.

결과 : 복령약침군(125mg/kg 복령약침군 및 250mg/kg 복령약침군)에서 insulin과 triglyceride, NEFA 수치가 유의하게 감소하였으며, 간 기능 효소수치인 sGOT가 감소하는 경향을 나타내었으나, 신장기능지수는 유의한 감소를 나타내지 않았다. 특히 250mg/kg 복령약침군에서 streptozotocin 투여로 인한 pancreatic islet의 형태학적 변성이 현저하게 개선되었다. Western blot 결과 JNK-2, P-JNK-2, P-JNK-1, ERK1/2 및 phosphorylated ERK1의 발현이 감소되었다.

결론 : 복령약침이 고인슐린혈증과 고지질질혈증을 개선시키고 streptozotocin에 의한 pancreatic islet의 파괴를 억제하며, 이는 inflammation-related transcription factor인 NF-kB와도 관련이 있는 것으로 판단된다. 향후 복령약침의 항당뇨 효과와 그 기전에 관한 추가 연구가 필요할 것으로 사료된다.

핵심단어 : *Poria cocos*, streptozotocin, diabetes mellitus, hyperinsulinemia

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Ⅰ. Introduction

From recent studies, diabetets mellitus is thought of as a group of metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both, associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels[1]. Type I diabetes results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas, leading to absolute insulin deficiency[2].

In the progress of development of diabetes mellitus, destruction of insulin-producing pancreatic β-cells are mediated by infiltration of CD4+ and CD8+ T cells and macrophages[3], and local production of inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-ɣ[4]. Particularly in spontaneous animal models such as the non-obese diabetic mouse and the Bio-breeding rat, macrophages and dendritic cells are the first cells that appear at the periphery of pancreatic ducts and islets of Langerhans[5].

Poria cocos (*P. cocos*), which is known as Fu Ling, is often used in Korean traditional medicine. Dried sclerotia of *P. cocos* is prescribed to cause diuresis for edema, oliguria, to invigorate the spleen and calm the mind for loose stools, and to treat diarrhea, restlessness, insomnia[6]. Recent studies reported that *P. cocos* exhibited anti-inflammatory[7], anti-emetic[8], renal protective effect from inflammation[9], anti-spastic, anti-ulcerative, and reducing gastric hyperacidity effect[10], and inhibitory effect on tumor promotion[11].

In this study, analysis of the blood parameters and the inflammatory protein expression, and histological observation of the pancreas in the rodent streptozotocin-induced diabetic model were conducted to evaluate anti-diabetic, pancreas protective effect of *P. cocos*.

Ⅱ. Materials and Methods

1. Materials

P. cocos used in this study was purchased from Dukhyundang (South Korea), a reputable Korean herb vender and identified and authenticated with the assistance of Department of Herbology, College of Oriental Medicine, Kyung Hee university. *P. cocos* (600g) was extracted by boiling in distilled water (6,000ml) for 4 hours in a decocting machine, and then filtered through filter papers (Whatman, England). The crude water extract solution was concentrated with a vacuum rotary evaporator under low pressure, and then the residue was lyophilized in a freezing dryer.

2. *In vivo* experiment

1) Animals

Male SD (Sprague-Dawley) rats were obtained at 5 weeks of age from Orient Company (Seoul, Korea) and acclimated for 1 week before being randomly assigned into the groups. The animals were housed in polypropylene cages with steel grid tops, and fed with water and standard rodent chow (Lab diets, Richmond, USA) *ad libitum*. The animal rooms were controlled at a 12 : 12-hours light-dark cycle (8 : 00 am to 8 : 00 pm), a temperature of 23± 2℃, and a humidity of 50±10% throughout the acclimatization and experimental periods.

The animals were randomly divided into four groups; control group (citrate buffer only), STZ (Streptozotocin only), and herbal acupuncture groups (P125 and P250). Herbal acupuncture groups were injected subcutaneously at left and right *Sinsu* (BL_{23}) alternately on exactly the same time every day with either 125mg/kg or 250mg/kg of the *P. cocos* (P125 and P250). After 1 week of administration of *P. cocos* herbal acupuncture, in order to induce type I diabetes, 65mg/kg of Streptozotocin (STZ) was dissolved in 0.1 M sodium citrate buffer (pH 4.5) and injected intraperitoneally. After 72 hours

from intraperitoneal injection of STZ, animals with serum glucose level above 300mg/dL were selected and continuously administrated with each herbal acupuncture for 2 weeks. The animals assigned as a control group were only injected intraperitoneally with citrate buffer. The experiments were conducted in accordance with internationally-accepted experimental protocols for laboratory animal use and care as found in the U.S. guidelines.

2) Blood Analysis

At the end of the study, the animals were sacrificed for blood analysis after 12 hours of fasting period. The blood samples withdrawn from heart were centrifuged at 5,000rpm for 15 minutes, and the plasma was stored at -70℃ until assay. The plasma glucose concentration was determined using the glucose oxidase method (Trinda medhod), and the spectrophotometric analysis was determined using the UV spectrophotometer (U-3210, Hitachi ™, Japan). The plasma insulin concentration was measured by ELISA reader (Labsystems, Finland), using the Rat insulin ELISA kit (Shibayagi, Japan). Plasma triglyceride (TG), total cholesterol (TC) were determined using commercially available kits (Asan Pharmaceutical Co, Korea), and the plasma nonesterified fatty acid (NEFA) concentrations were assayed by an enzymatic colorimetric method (Shinyang Chemical Co, Korea). sGOT (serum Glutamic Oxaloacetic Transaminase), sGPT (serum Glutamic Pyruvic Transaminase), ALP (alkaline phosphatase), BUN (Blood Urea Nitrogen), CRE (creatinine), indices of functional enzymes of liver and kidney, were analyzed using the International Federation of Clinical Chemistry (IFCC) method with a SMAR-TLAB (STANBIO Co, USA).

3) Histopathology

Wedges of pancreatic tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into thin sections (5㎛), and then deparaffinized with xylene, hydrophilized by 100%, 95%, 90%, 80%, 70% of alcohol, Canada embalsamed and then stained with hematoxylin and eosin (H&E). The slides were observed through light microscope (Olympus, Japan).

4) Protein analysis and western blot

The pancreatic tissues which were equivalently weighed and homogenized in PRO-PREP Solution, were centrifuged at 5,000rpm. The protein concentration was determined with Bio-Rad Dc protein assay method. For immunoblotting, the protein samples were subjected to 10% SDS-PAGE, electrotransferred to nitrocellulose paper, and blocked with 5% skim milk solution before incubating with primary antibody in PBS buffer solution for more than 12 hours. The samples were then washed with TPBS three times and subsequently incubated with secondary antibody attached with horseradish peroxidase for 1 hour, and finally washed with TPBS three times. To detect proteins, a chemiluminescent signal was developed using the ECL kit.

3. *In vitro* experiments

1) Cell culture

Rat insulinoma (RINm5F) cell strain was purchased from ATCC (American Type Culture Collection). Stocks of RINm5F cells were maintained in RPMI1640 medium containing 10% fetal bovine serum (FBS), penicillin (100U/ml), streptomycin (100U/ml), glutamine) (Gibco) supplemented with 2.5㎍/ml of amphotericin B in the CO_2 cultivator at 37℃, 5% CO_2.

2) Analysis of cellular toxicity

Cellular toxicity of aqueous extraction of *P. cocos* was analyzed using 3-(4,5-dimethlthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay method at the concentration of 0, 0.0625, 0.125, 0.25, 0.5 and 1mg/ml. RINm5F stain cells (1×10^5 cell/well) were grown in the 96 well plate and treated with the varied concentrations of *P. cocos* extract for 24 hours. After 30 minutes incubation at 37℃ with additional 20㎕ of MTS solution, cellular toxicity was analyzed with OD value spectrophotometrically

measured at 450nm.

3) Western blot

After 1 x 10^6 cells/well of RINm5F cells in 6 wells were acclimatized for 1 day, and then administered with STZ (10mM) and *P. cocos* aqueous extration (0.125mg/ml, 0.25mg/ml, 0.5mg/ml) for 24 hours, inflammation-related protein such as iNOS was measured.

4. Statistical analysis

All data were expressed as a mean±S.E. Comparisons of data were done by Dunnett's two-tailed *t* test. Mean values were considered significantly different when $p<0.05$.

Ⅲ. Results

1. Fasting plasma glucose, and insulin resistance index

The fasting plasma glucose, insulin, TG, TC, NEFA, sGOT, sGPT, ALP, BUN and CRE level were compared between groups (Table 1). Fasting insulin, TG, and NEFA level in the P125, P250 herbal acupuncture treatment groups showed significant differences when compared to STZ group. There were no significant differences in the plasma glucose, TC levels between groups. sGOT level in the P125, P250 herbal acupuncture treatment groups showed a significant difference, but there were no significant differences in the BUN and CRE level between the groups(Table 1).

2. Morpholgy of pancreas

Morphology of pancreatic islets from the control

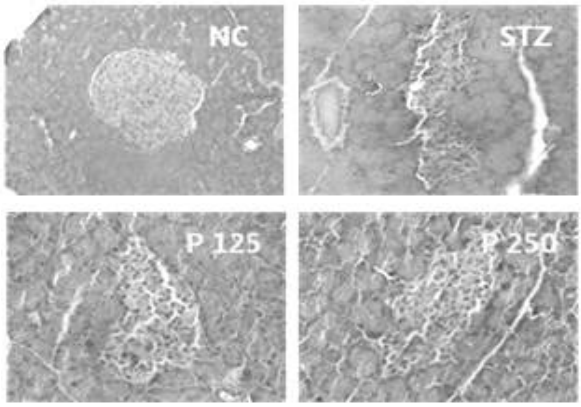

Fig. 1. Protective effect of *P. cocos* herbal acupuncture at *Sinsu* (BL_{23}) on pancreatic islets from STZ-induced destruction

Microscopic views on the sections of the pancreas obtained from NC, STZ, P125 and P250. H&E, magnification×200.

NC : citrate buffer.

STZ : streptozotocin intraperitoneally injected.

P125 : 125mg/kg of the *P. cocus* herbal acupuncture.

P250 : 250mg/kg of the *P. cocus* herbal acupuncture.

Table 1. Effects of *P. cocos* Herbal Acupuncture on Plasma Parameters

	NC	STZ	P125	P250
Glucose (mg/dL)	163.9±9.4	378.0±11.8	364.3±26.7	386.6±35.2
Insulin (ng/ml)	2.18±0.17	0.65±0.03	0.94±0.10**	1.11±0.12**
TG (mg/dL)	69.2±3.9	249.7±60.1	190.4±44.9*	113.5±22.0*
TC (mg/dL)	64.5±2.8	78.6±5.8	67.9±5.3	71.5±5.8
NEFA (uEq/L)	469±24	806±85	564±67*	549±59*
sGOT (U/L)	1 19.6±34.7	208.2±26.5	124.5±31.9**	145.4±32.2**
sGPT (U/L)	42.5±7.3	86.6±18.0	86.0±7.7	103.5±14.2
ALP (U/L)	1 83.5±29.8	317.6±37.5	418.5±44.3	360.8±78.7
BUN (mg/dL)	14.4±1.4	35.8±6.9	42.4±8.6	33.9±7.5
CRE (mg/dL)	1.014±0.1	0.91±0.1	0.91±0.1	0.84±0.1

Values represent the mean±SE (n=6). Plasma parameters were analyzed in plasma samples obtained from blood of 12 hr fasted mice.

* : $p<0.05$. ** : $p<0.01$. *** : $p<0.001$ vs. STZ group.

and the *P. cocos* herbal acupuncture in SD rats after 3 weeks of treatment was shown in Fig. 1. Morphology of islets of the rats of P250 group was remarkably improved when compared to those of STZ group (Fig. 1).

3. Effect of *P. cocos* on the expression of inflammation-related proteins in pancreas

Changes of expression of inflammation-related factors (extracellular signal-regulated kinase (ERK), c-jun N terminal kinase (or, stress-activated protein kinase)) analyzed by Western blot in SD rats after 3 weeks of treatment was shown in Fig. 2. The expressions of JNK-2 and P-JNK-2 were significantly decreased when compared to STZ group. There were no significant differences in the expression of JNK-1 between groups, however the expression of P-JNK-1 was significantly decreased when compared to STZ group. The expression of ERK1/2 was decreased when compared to STZ group. There were no significant differences in the expression of phosphorylated ERK2 between groups; however, phosphorylated ERK1 was decreased (Fig. 2, 3).

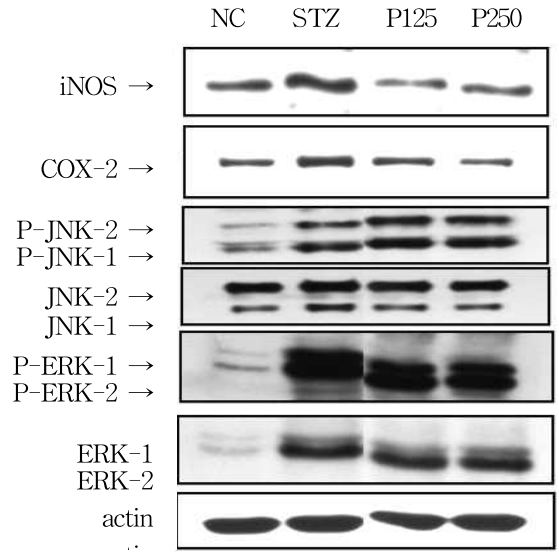

Fig. 2. Effects of *P. cocos* herbal acupuncture at *Sinsu* (BL_{23}) on iNOS, COX-2, TNF-a and phosphorylation of ERK1/2 and JNK 1/2 in STZ-induced rat pancreas

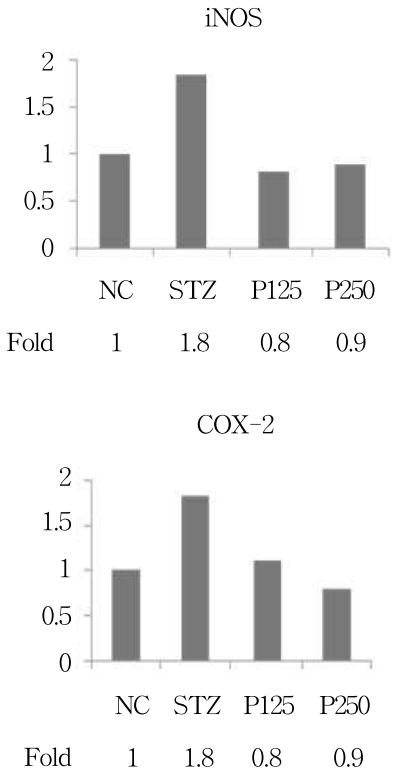

Fig. 3. Effects of *P. cocos* herbal acupuncture solution on cytotoxicity measured by MTS assay

IV. Discussion

Type I diabetes mellitus is an autoimmune disease characterized by selective destruction of pancreatic β-cells and related with insulitis by infiltration of T cells and macrophages and local production of inflammatory cytokines such as IL-1 β, TNF-α, IFN-γ[1-4]. In type I diabetes, β-cell mass is reduced by 70−80% at the time of diagnosis. Because of the variable degree of insulitis and absence of detectable β-cell necrosis, it was suggested that β-cell loss occurs slowly over years[12]. The rate of β-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults)[13].

Autoimmune destruction of β-cells has multiple genetic predispositions and is also related to environmental factors that are still poorly defined.

Although patients are rarely obese when they present with this type of diabetes, the presence of obesity is not incompatible with the diagnosis. These patients are also prone to other autoimmune disorders such as Grave's disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, and pernicious anemia[1]. Markers of the immune destruction of the β-cell include islet cell autoantibodies (ICAs), autoantibodies to insulin (IAAs), autoantibodies to glutamic acid decarbo xylase (GAD65), autoantibodies to the tyrosin phosphatases IA-2 and IA-2β. One and usually more of these autoantibodies are present in 85-90% of the individuals when fasting hyperglycemia is initially detected[14]. β-cell death in the course of insulitis is probably caused by direct contact with activated macrophages and T-cells, and/or exposure to soluble mediators secreted by these cells, including cytokines, nitric oxide (NO), and oxygen free radicals[15]. In the insulitis lesion in type I diabetes, invading immune cells produce cytokines, such as IL-1, TNF-α, and IFN-ɣ, and induce β-cell apoptosis via the activation of β-cell gene networks under the control of the transcription factors NF-B and STAT-1. The execution of β-cell death occurs through activation of mitogen-activated protein kinases, via triggering of ER stress and by the release of mitochondrial death signals[16].

STZ (N-methyl-N-nitrosoureido D-glucosamine) derived originally from *Streptomyces achromogenes*[17] is a natural product that selectively kills pancreatic β-cells, and is widely used to generate insulin-dependent diabetes[18] in mouse models or treat pancreatic tumors[19]. Intraperitoneal administration of STZ is one of efficacious method to induce diabetes in rodents[20], and assumed that it is mediated by two mechanisms; the chemical poison model and the O-GlcNAc-dependent model. The first is linked to the N-nitrosourea group act as a DNA alkylating agent and/or nitric oxide donor[21]. Several studies propose that STZ induces apoptosis by inhibiting O-GlcNAcase, an enzyme that, together with O-GlcNAc transferase, is important for dynamic intracellular protein O-glycosylation[22]. STZ action in β-cells is accompanied by characteristic alteration in blood insulin and glucose concentrations. These changes reflect abnormalities in β-cell function (glucose oxidation, insulin biosynthesis and secretion, responsiveness to glucose)[23].

Recently, studies for treatments of Type I diabetes mellitus have been done using various modalities such as acpuncture, moxibustion, electroacupuncture, herbal acupuncture and laser acupuncture[24].

Of them, previous studies show the effect of *Cinnamomi Ramulus*[25,26], *Ginseng Radix*[27], *Lycii Radicis Cortexi*[28] in reducing hyperglycemia. Also, *Acanthopanacis Cortex*[29], *Astragali Radix*[30] intravenous herbal acupuncture have been proven to be effective in protecting intradermal cells by decreasing the filtration rate of proteinuria and intradermal cell count in plasma and urine, and thereby maintaining renal function. There have been few studies so far; however, about preventing β-cell damage mechanism of Type I diabetes mellitus using *P. cocos* herbal acupuncture. In this study, we found that *P. cocos* herbal acupuncture on streptozotocin-induced diabetic rat can prevents β-cell damage and thus protect pancreas.

Dried sclerotia of *P. cocos*, *Fu ling* is prescribed to cause diuresis for edema, oliguria, to invigorate the spleen and calm the mind for loose stools, and to treat diarrhea, restlessness, insomnia[6]. *P. cocos* consists of 90% β-glucan and 10% various terpenes by dry weight. Three major compounds were isolated and identified as pachymic acid, 3β-hydroxylanosta-7,9(11),24-trien-21-oic acid, and dehydroeburicoic acid[31]. Recent studies reported that *P. cocos* exhibited anti-inflammatory[7], antiemetic[8], renal protective effect from inflammation[9], anti-spastic, anti-ulcerative, and reducing gastric hyperacidity effect[10], and inhibitory effect on tumor promotion[11].

Sato *et al.* demonstrated that the triterpene acid compound dehydrotrametenolic acid isolated from dried sclerotia of *P. cocos* promotes adipocyte differentiation *in vitro* and acts as an insulin sensitizer *in vivo*. Dehydrotrametenolic acid reduced hyperglycemia in obese hyperglycemic *db/db* mice and acted as an insulin sensitizer as indicated by the

results of the glucose tolerance test[32]. However, Son *et al.* reported that aqueous extract of *P. cocos* had some decreasing effect on the level of blood glucose in the fructose rich diet fed hyperglycemic rats and cholesterol rich diet fed hyperlipidemic rats but had no diminishable effects on the glucose tolerance, so the anti-diabetic effect was not dependent on insulin secretions[33].

Several points are in discord on the anti-diabetic effect of *P. cocos* and its mechanisms between reports because there is some differences of its experimental protocols. Sato *et al.* for example, used *db/db* C57BLKS/J mice (genotype +/+) orally administered dehydrotrametenolic acid isolated from *P. cocos* for 14 days to estimate the anti-diabetic effect by measuring the levels of blood glucose, serum insulin, and the glucose tolerance test, but Son et al. measured blood glucose level in the fructose and cholesterol - rich diet fed hyperglycemic SD rats with orally administering crude extract of *P. cocos* for 3 weeks. And the insulin sensitizing effect was estimated in the SD rats intravenously injected with STZ by the glucose tolerance test, without measuring the serum insulin level.

In this study, with decreased plasma insulin level, lipid profile (TG, NEFA) of the P125, P250 herbal acupuncture treatment group was also reduced. *P. cocos* herbal acupuncture also improved liver profile (sGOT). These results suggest that *P. cocos* herbal aqueous extract inhibits hyperinsuline mia induced by STZ-induced diabetes.

With 3 weeks of treatment, *P. cocos* herbal acupuncture prevented the morphological deterioration induced by STZ. These results suggest that *P. cocos* aqueous extract has preventative effect from STZ-induced pancreatic islet destruction.

Furthermore, in this study, the significant decrease of expression of inflammation-related factors (JNK-2, P-JNK-2, and P-KNK-1) were observed, and also the decrease of expression ERK1/2 and phosphorylated ERK1 were also detected. These results suggest that *P. cocos* aqueous extract protects the β-cells from STZ-induced pancreatic islet destruction and the protective effect of *P. cocos* aqueous extract is related with NF-kB, the inflammation-related transcription factor.

Further study on the anti-diabetic effect of *P. cocos* and its mechanisms is needed.

Ⅴ. Conclusion

In this study, *P. cocos* aqueous extract was subcutaneously injected at *Sinsu*(BL_{23}) for 3 weeks to STZ-induced diabetic rodent model, and the plasma glucose, insulin, TG, TC, NEFA, sGOT, sGPT, ALP, BUN, CRE level were examined. Also, histological examination and quantitative analysis of expression of inflammation related proteins were conducted. As shown in the results, *P. cocos* herbal acupuncture improved hyperinsulinemia and hyperlipidemia, and protected pancreatic destruction induced by STZ. And the protective effect of *P. cocos* aqueous extract is related to NF-kB, the inflammation-related transcription factor.

Ⅵ. References

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