

Icariin prevents cytokine-induced β -cell death by inhibiting NF- κ B signaling

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Abstract. The loss of insulin secretion in type I diabetes mellitus (T1DM) is caused by autoimmune-mediated destruction of insulin-producing pancreatic β -cells. Inflammatory cytokines and immune cell infiltration activate oxidative and endoplasmic reticulum (ER) stress, resulting in reduced β -cell viability. The current pharmacological agents used to control blood glucose have a limited effective duration and are accompanied by strong side effects. Blocking the inflammatory and immune responses that cause the β -cell damage has been investigated as a novel therapeutic approach to control T1DM. Icariin is a flavonoid component of Chinese medicinal herbs that has anti-inflammatory effects *in vitro* and *in vivo*. The results of the present study revealed that icariin abrogates the pro-apoptotic effect of inflammatory cytokines and significantly suppresses the activation of nuclear factor (NF)- κ B in rat pancreatic β -cell lines. The present study may provide a basis for the potential use of icariin as a therapeutic agent for T1DM.

Introduction

The incidence of diabetes mellitus (DM), a complex metabolic disorder associated with defective insulin secretion and activity, has been increasing worldwide over the past 20 years (1,2). According to the World Health Organization estimation, ~7% of the world's adult population is diabetic and

the diabetic population is likely to increase to ≥ 300 million by the year 2025 (3). Type 1 DM (T1DM) is an organ-specific autoimmune disease associated with failure to distinguish self- from non-self-antigens (4). It is caused by T cell-mediated destruction of insulin-producing pancreatic β -cells (4). The incidence of T1DM is increasing steadily by 3% annually and has a concordance rate of 40-60% for monozygotic twins (4). As such, environmental and genetic factors may contribute to disease onset (5-7). Currently, insulin therapy is the primary treatment for T1DM. However, tight glycemic control is difficult to achieve in a number of patients, leading to long-term vascular damage associated with kidney failure, heart disease, retinopathy and neuropathy (8). Recent advances in pancreatic islet transplantation and partial or whole pancreas transplantation represent alternate treatment options for T1DM (9). However, due to the limited number of organs available for transplant, this approach is not widely used (9). Since β -cell damage is crucial to the development of T1DM, treatments that are able to prevent β -cell damage may slow disease progression.

Pancreatic β -cell damage is known to be mediated by the immune response (10-12). Previous studies have suggested that inflammatory cytokines and immune cell infiltration activate oxidative and endoplasmic reticulum (ER) stress and damage β -cell viability (10-16). In the early stages of disease, the infiltration of inflammatory cells promotes the release of cytokines, including interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) (15-16). IL-1 β , alone or in combination with TNF- α or IFN- γ , upregulates the expression of inducible nitric oxide synthase (iNOS) and promotes the generation of nitric oxide (NO) in pancreatic islets (17,18). Excessive NO production leads to dysfunctions of mitochondrial metabolism, protein modification and DNA cleavage, which may contribute to the impairment of insulin secretion and triggering β -cell death (19).

Considering the inflammatory nature of T1DM, it is plausible that anti-inflammatory agents may have potential as anti-DM drugs. Icariin is a naturally occurring flavonoid isolated from traditional Chinese medicinal herbs of the *Epimedium* genus (20). The compound has been revealed to have anti-inflammatory, antidepressant, male reproductive, anti-neoplastic, bone-healing and neuroprotective effects (20). Early

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in vivo and *in vitro* studies revealed that icariin acts as a natural anti-inflammatory drug via multiple mechanisms targeting pro-inflammatory cytokines (TNF- α and IL-6), inflammatory mediators (NO) and adhesion molecules (CD11b) (21,22). Xu *et al* (23) reported that icariin activates the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway to ameliorate lipopolysaccharide (LPS)-induced acute inflammatory responses. The known anti-inflammatory effects of icariin suggest that it may inhibit inflammation-induced β -cell death. The aim of the present study was to use rat pancreatic β -cell lines as an *in vitro* model to investigate the role of icariin. The results suggest that icariin inhibits cytokine-induced NF- κ B activation and prevents β -cell death.

Materials and methods

Cell culture. Rat pancreatic β -cell RINm5F cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 mM glutamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 1% non-essential amino acids (Sigma-Aldrich; Merck KGaA), 100 U/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA) at 37°C in an atmosphere containing 5% CO₂. Icariin was purchased from Sigma-Aldrich (Merck KGaA). Rat IL-1 β and IFN- γ proteins were obtained from R&D Systems (Minneapolis, MN, USA).

MTT assay. MTT (Sigma-Aldrich; Merck KGaA) was used to determine cell viability according to the manufacturer's protocols. Briefly, 5 ml MTT solvent (Beyotime Institute of Biotechnology, Haimen, China) was used to dissolve 25 mg MTT to form an MTT solution at 5 mg/ml. A total of 10 μ l MTT solution was added to each well and incubated for 4 h at 37°C in an incubator. Subsequently, 100 μ l formazan solution (Beyotime Institute of Biotechnology) was added for 4 h at 37°C. The optical density of viable cells was measured using a microplate reader (BMG Labtech GmbH, Ortenburg, Germany) at a wavelength of 570 nm.

NO measurement. Biologically synthesized NO is quickly oxidized to form nitrite and nitrate in aqueous solutions (19). Therefore, detecting the nitrite concentration in cell-free culture supernatants using a colorimetric assay may be indicative of NO generation. In brief, RINm5F cells (5x10⁶) or 30 islets were treated with the 5 or 10 μ M concentrations of icariin for 3 h, prior to being treated with IL-1 β (1 U/ml) and IFN- γ (100 U/ml) for 24 h. Subsequently, 100 μ l aliquots of culture supernatant were incubated at room temperature for 5 min with 100 μ l modified Griess reagent in a 1:1 mixture of 1% sulfanilamide in 30% acetic acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 60% acetic acid (Beyotime Institute of Biotechnology). The absorbance was measured at 540 nm. The NO concentration was calculated from the linear standard curve of serial dilutions of sodium nitrite in a working medium.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured

cells using TRIzol reagent (Thermo Fisher Scientific, Inc.). The primer for iNOS was synthesized based on the following previously published sequences (24): Forward, 5'-GAATCT TGGAGCGAGTTGTGG-3' and reverse, 5'-AGGAAGT AGGTGAGGGCTTGG-3'. First-strand cDNA was obtained using Super M-MLV Reverse transcriptase (BioTeke Corporation, Beijing, China). Reverse transcription was performed at 42°C for 15 min and 72°C for 2 min according to the manufacturer's protocols. PCR was performed using SYBR-Green master mix (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The following thermocycling conditions were used: Predenaturation at 95°C for 30 sec followed by 40 cycles of amplification at 95°C for 5 sec and annealing and extension at 60°C for 30 sec. GAPDH was used to normalize iNOS mRNA expression. GAPDH forward, 5'-GATGACCTTGCCCACAGCCT-3' and reverse, 5'-ATCTCTGCCCCCTCTGCTGA-3'. The 2^{- $\Delta\Delta$ C_q} method was used to quantify data (24). ABI Prism 7000 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to analyze data.

Western blotting. Following treatment, proteins were extracted from RINm5F cells using a Nuclear and Cytoplasmic Protein Extraction kit (cat. no. P0027; Beyotime Institute of Biotechnology). Protein concentrations were determined using an Enhanced BCA Protein Assay kit (cat. no. P0010S; Beyotime Institute of Biotechnology). A total of 20 μ g/lane was separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked using Blocking Buffer (cat. no. P0023B; Beyotime Institute of Biotechnology) for 2 h at room temperature. Proteins were probed using specific primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 1 h. Specific primary antibodies against pro-caspase-3 (ab44976; 1:500), cleaved caspase-3 (ab13847; 1:500) and cleaved poly ADP-ribose polymerase (PARP; ab32064; 1:2,000) were purchased from Abcam (Cambridge, UK). Secondary antibodies against β -actin (ab8227; 1:2,000) and Larmin A (ab26300; 1:1,000) used in this study were horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG or anti-mouse IgG-HRP (Beyotime Institute of Biotechnology). β -actin and Larmin A were used as internal controls to normalize results. Signals were monitored using a chemiluminescent substrate (KPL, Inc., Gaithersburg, MD, USA). Following electrophoresis, gray values were analyzed using Quantity One v4.4.0.36 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Caspase-3 activity. The activity of caspase-3 was conducted using a commercial ELISA kit (cat. no. HC079; Shanghai Gefan Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's protocols. In brief, cells (1x10⁶) were resuspended in 50 μ l lysis buffer (Shanghai Gefan Biotechnology Co., Ltd.) and incubated for 1 h in an ice bath. The supernatant was collected following centrifugation for 10 min at 800 x g at room temperature, following which a colorimetric reagent was added and incubated for 4 h at 37°C. The colorimetric product was monitored using an ELISA reader at a wavelength of 405 nm.

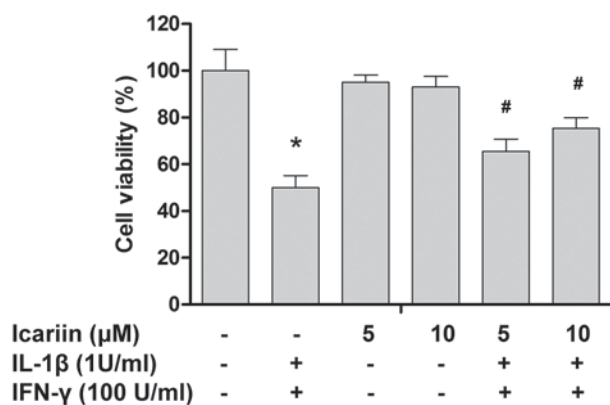


Figure 1. Icariin prevents cytokine-induced cell death in RINm5F cells. RINm5F cells were treated with icariin at 5 or 10 μ M with or without IL-1 β and IFN- γ stimulation and cell viability was assessed using an MTT assay. * P <0.05 vs. control and # P <0.05 vs. IL-1 β +IFN- γ . IL, interleukin; IFN, interferon.

Apoptosis detection using flow cytometry. A total of 1×10^6 cells were washed with PBS and resuspended in binding buffer containing Annexin V-APC and propidium iodide, and incubated at 20-25°C for 10-20 min (Beyotime Institute of Biotechnology). The samples were analyzed using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of apoptotic cells in a 10,000-cell cohort was determined using flow cytometry.

NF- κ B P65 activity. Following treatment, nuclear extracts were isolated using the Nuclear Extract kit according to the manufacturer's protocols (Active Motif, Carlsbad, CA, USA; cat. no. 40010). The activity of NF- κ B p65 was assessed using an ELISA kit (cat. no. 40596; Active Motif).

Statistical analysis. Values are presented as the mean \pm standard deviation. Statistical comparisons between cell lines were performed using one-way analysis of variance, followed by Dunnett's t-test. GraphPad Prism 7.03 software (GraphPad Software Inc., La Jolla, CA, USA) was used to analyze experimental data and a P <0.05 was considered to indicate a statistically significant difference.

Results

Icariin prevents cytokine-induced loss of cell viability. To assess the therapeutic potential of icariin in rat pancreatic β cells, the viability of cultured RINm5F cells was initially examined. As presented in Fig. 1, treatment with icariin up to 10 μ M did not result in a significant loss of cell viability. Next, whether icariin protected RINm5F cells from cytokine toxicity was investigated. Treatment with cytokines IL-1 β and IFN- γ significantly reduced the cell viability to $49.9 \pm 5.2\%$ of the control value (Fig. 1). Pretreatment with icariin significantly abrogated the cytotoxic effects of cytokines on RINm5F cells in a concentration-dependent manner.

Icariin prevents cytokine-induced NO production. NO production was significantly increased following 24 h

treatment with cytokines (Fig. 2A). However, the cytokine-induced NO production was effectively inhibited by treatment with 10 μ M icariin (Fig. 2A). To investigate the underlying mechanisms responsible for the effects of icariin, RT-qPCR and western blotting were performed to measure the expression of iNOS at the mRNA and protein level, respectively. Treatment with IL-1 β and IFN- γ significantly increased the expression of iNOS, while icariin treatment significantly ameliorated this increase at the mRNA and protein level (Fig. 2).

Icariin prevents cytokine-induced apoptosis. Cytokines are able to promote β -cell death through apoptosis and necrosis (25). Caspase-3 serves a pivotal role in the apoptotic signaling pathway, and so the activation status of caspase-3 was assessed in the present study. Treatment with IL-1 β and IFN- γ increased the activity of caspase-3 and cell apoptosis in RINm5F cells, while icariin effectively reversed these effects (Fig. 3). The activation of apoptotic signaling was also confirmed by western blotting (Fig. 3B). Cleaved caspase-3 is the main marker of cell apoptosis (26), and so its expression was assessed. As presented in Fig. 3B, IL-1 β and IFN- γ were able to activate caspase-3 and increase the cleavage of PARP in RINm5F cells, while treatment with icariin reduced cleaved caspase-3 and cleaved PARP levels in cytokine-stimulated cells.

Icariin suppresses the cytokine-induced activation of NF- κ B. NF- κ B is a key transcription factor that induces iNOS and regulates subsequent NO production (27). The results of a previous study by our group demonstrated that NF- κ B was activated by cytokines or oxidative stress (28). Based on this, it was investigated whether icariin affects the cytokine-induced activation and translocation of NF- κ B from the cytosol to the nucleus in RINm5F cells. NF- κ B and the nuclear translocation of p65, a key subunit of the NF- κ B complex, were significantly promoted by treatment with IL-1 β and IFN- γ compared with the control (Fig. 4). In contrast, icariin pretreatment markedly suppressed the cytokine-stimulated activation and nuclear translocation of NF- κ B. In summary, these data suggest that icariin may downregulate iNOS expression via inhibiting the cytokine-stimulated activation of NF- κ B.

Discussion

Icariin is a biologically active flavonoid with a favorable therapeutic profile in metabolic syndrome (29,30). Notably, icariin has been reported to ameliorate streptozocin-induced rat diabetic retinopathy and nephropathy (31,32). A previous study demonstrated that icariin could serve as a peroxisome proliferator-activated receptor α agonist, which activates gene expression associated with lipid metabolism in the liver to contribute towards diabetes management (33). In the present study, it was revealed that icariin is able to prevent cytokine-induced β -cell death, which is an important cause of T1DM.

Inflammation is the primary cause of T1DM as well as a direct cause of a number of diabetic complications (34). An acute, intense inflammatory response triggers T1DM

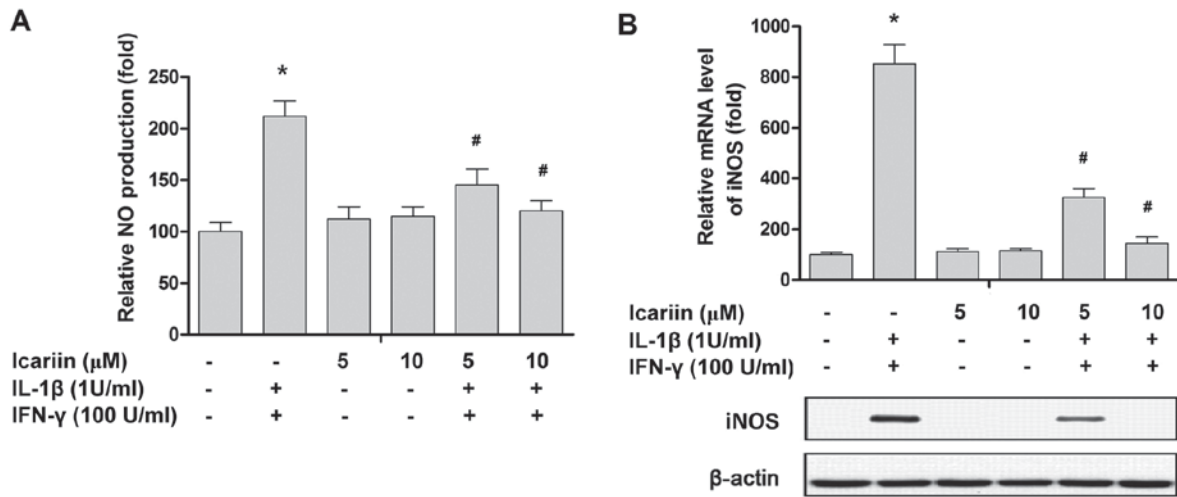


Figure 2. Icariin prevents cytokine-induced NO production in RINm5F cells. RINm5F cells were treated with 5 or 10 μ M icariin with or without IL-1 β and IFN- γ stimulation. (A) NO production and (B) iNOS levels were detected using a colorimetric assay, reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. *P<0.05 vs. control and #P<0.05 vs. IL-1 β +IFN- γ . NO, nitric oxide; IL, interleukin; IFN, interferon; iNOS, inducible nitric oxide synthase.

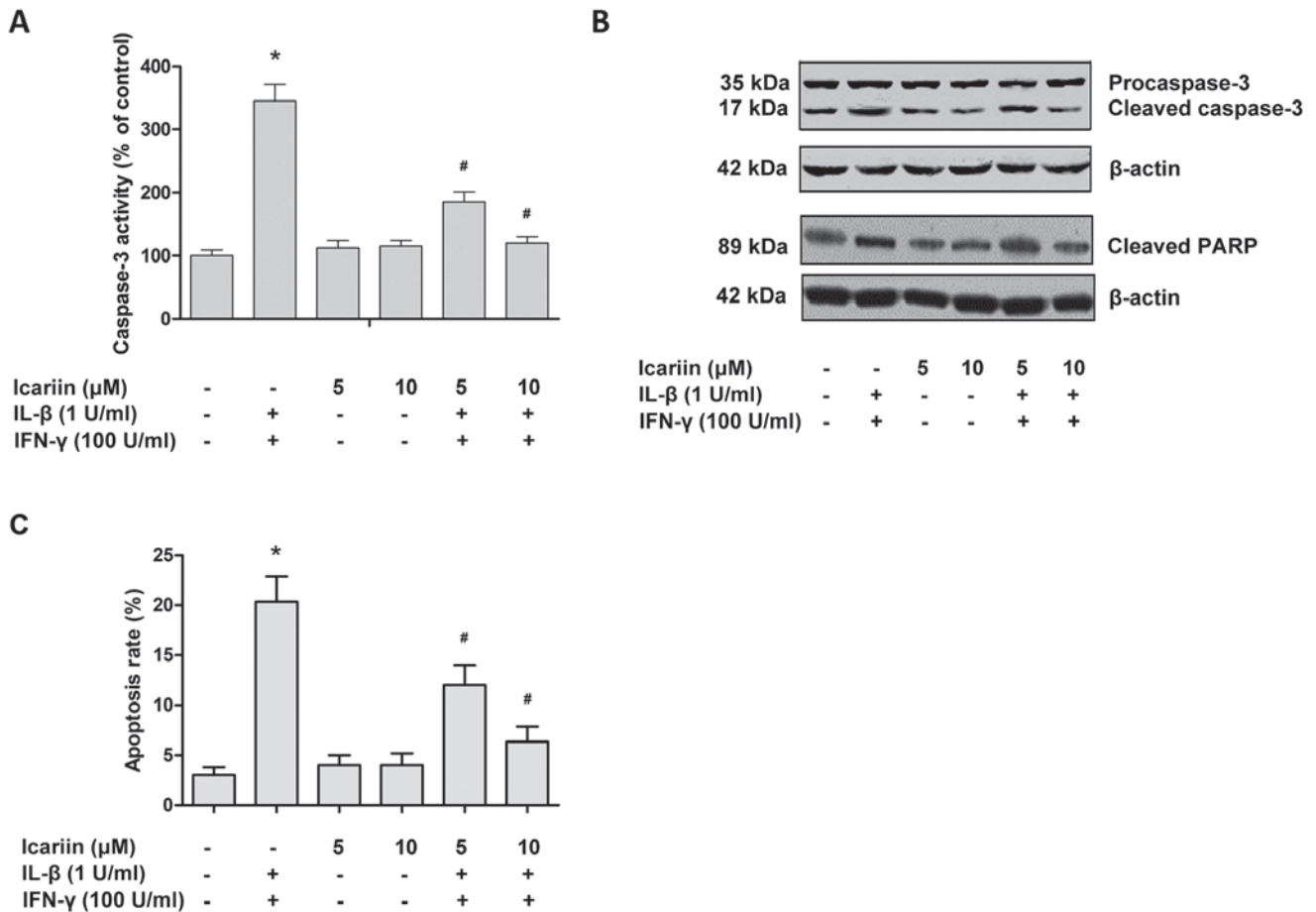


Figure 3. Icariin inhibits cytokine-induced caspase-3 activation and PARP cleavage as well as cell apoptosis in RINm5F cells. RINm5F cells were treated with icariin at 5 or 10 μ M with or without IL-1 β and IFN- γ stimulation. Subsequently, caspase-3 activation and PARP cleavage were determined using (A) ELISA and (B) western blotting assays. (C) Cell apoptosis was detected using flow cytometry with Annexin V-Allophycocyanin and propidium iodide staining. *P<0.05 vs. control, #P<0.05 vs. IL-1 β +IFN- γ . PARP, poly ADP-ribose polymerase; IL, interleukin; IFN, interferon.

through lymphocyte-mediated destruction of pancreatic β cells. A chronic state of low-grade inflammation persists within in the body, which is periodically exacerbated by

hyperglycemic fluctuations (34-35). Increased inflammation markers (35), immune activation (36) and oxidative stress have been recorded in patients with T1DM (37,38).

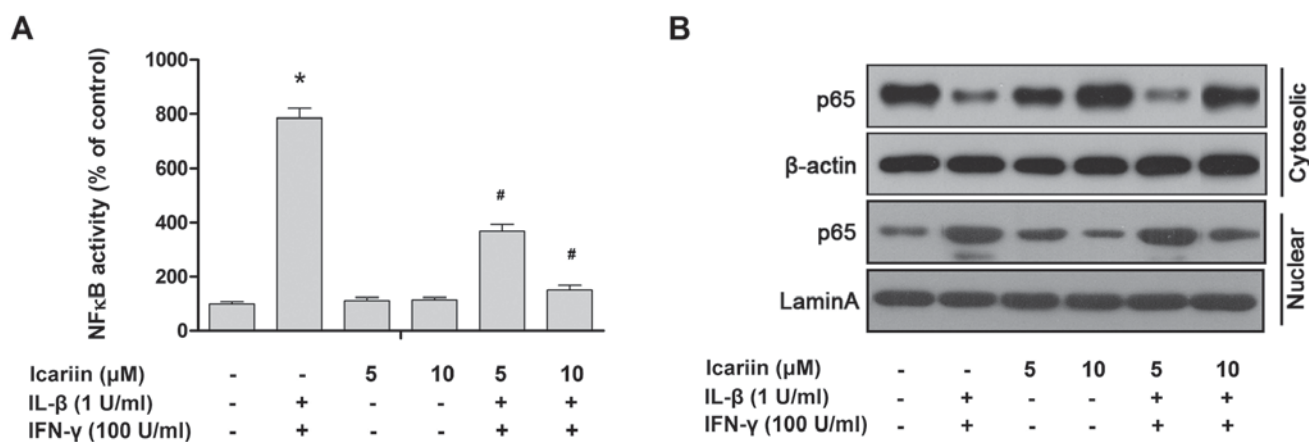


Figure 4. Icariin inhibits cytokine-induced NF- κB activation in RINm5F cells. RINm5F cells were treated with icariin at 5 or 10 μM with or without IL-1 β and IFN- γ stimulation. (A) NF- κB activation and (B) p65 nuclear localization were further analyzed using ELISA and western blotting, respectively. * $P < 0.05$ vs. control, # $P < 0.05$ vs. IL-1 β +IFN- γ . NF, nuclear factor; IL, interleukin; IFN, interferon.

It has therefore been hypothesized that anti-inflammatory agents may be an effective clinical treatment for patients with T1DM. A number of *in vivo* and *in vitro* studies have confirmed the anti-inflammatory effect of icariin, including in the brain, heart, bones and airways (22,23,39-50). The present study demonstrated that icariin prevents viability loss in rat pancreatic β cells, as well as suppressing cytokine-induced NO production and apoptosis activation. These results suggest that icariin may interfere with the inflammatory response and resulting pancreatic β cell death during T1DM.

Furthermore, a key factor in cytokine-induced pancreatic β -cell damage is NF- κB . *In vivo* studies of transgenic mice revealed that NF- κB inhibition is a protective mechanism against cytokine-induced apoptosis in pancreatic β -cells (28). In addition, the use of A20-overexpressing islets to abrogate NF- κB signaling during islet transplantation reduces the number of islets required to achieve euglycemia in diabetic recipients (51). Therefore, suppression of the NF- κB pathway may also be a novel strategy for delaying the progression of T1DM. The regulatory role of icariin on NF- κB has been reported, however it may vary between different cell types (20). Xu and Huang (52) demonstrated that icariin could increase the expression of endothelial NOS in human endothelial cells, which was implicated in the activation of NF- κB (53). In contrast, icariin was able to abrogate the effects of LPS on neuroinflammation, lung inflammation, osteoclast differentiation and bone resorption via decreasing NF- κB activity (23,49,54,55). It has also been reported that icariin inhibits NF- κB activity in a wide range of cancerous cells (56-59). The results of the present study demonstrated that icariin suppresses the cytokine-induced activation of NF- κB in rat pancreatic β cells. It is likely that icariin exerts cell-specific regulatory effects and only suppresses high levels of NF- κB activity in tumor cells or cells stimulated by inflammatory cytokines.

The results of the present study demonstrate that icariin abrogates the pro-apoptotic effect of cytokines and significantly suppresses NF- κB activation in rat pancreatic β -cells. Despite being used extensively as a model for the human pancreas, the physiology of rat pancreatic β cells does not

perfectly mimic that of primary cells (60). The RINm5F cells used in the present study have limitations in terms of glucose sensitivity, transport and phosphorylation (60,61). Therefore, experiments utilizing human pancreatic cells and *in vivo* analysis are required to confirm these findings. Nevertheless, the results of the present study suggest that icariin may have potential as a therapeutic agent against T1DM.

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Availability of data and materials

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

Authors' contributions

SZ and J-YY conceived the study, acquired data, interpreted the results and drafted the manuscript. JG made substantial contributions to the experiments and data analysis.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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