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Original Article

Berberine Induces Cell Apoptosis through Cytochrome C/Apoptotic Protease-Activating Factor 1/Caspase-3 and Apoptosis Inducing Factor Pathway in Mouse Insulinoma Cells*

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ABSTRACT **Objective:** To investigate apoptotic effects of berberine, a significant alkaloids component existing in *Rhizoma coptidis*, and its possible acting mechanism in insulinoma cells. **Methods:** Different concentrations of berberine were used to treat mouse insulinoma (MIN6) cells for various period of time. The viability and apoptosis of the cells were analyzed using methylthiazolyldiphenyl-tetrazolium bromide assay, flow cytometry and enzyme-linked immuno sorbent assay. Changes in the relating pro- and anti-apoptosis proteins were detected by western-blotting. **Results:** The half-maximal inhibitory concentration (IC_{50}) of berberine was 5.7 μmol/L on MIN6 cells viability for 16 h. Berberine caused a 20% reduction ($P<0.05$) in cell number after only 4-h incubation; which reached 50% after 24 h ($P<0.01$). Berberine treatment for 16 h significantly increased the level of DNA fragmentation. The flow cytometry showed the apoptotic rate increased 2.9- and 4.6-fold after treating with berberine (5 μmol/L) for 8 and 16 h, while 3- and 8.7-fold after 10 μmol/L treatment for 8 and 16 h ($P<0.01$). Berberine treatment dramatically elevated the expression ratio of Bax to Bcl-2. Meanwhile, berberine notably increased the apoptosis-inducing factors and cytochrome C transforming from the mitochondria to the cytoplasm. Apoptotic protease-activating factor 1 (Apaf-1) was subsequently activated after cytochrome C release. Furthermore, caspase-3 and poly adenosine diphosphate-ribose polymerase were also activated to trigger apoptosis cascade. **Conclusion:** High concentration (5 and 10 μmol/L) of berberine could induce the apoptosis of MIN6 cells through cytochrome C/Apaf-1/caspase-3 and apoptosis inducing factor (AIF) pathway.

KEYWORDS Chinese medicine, berberine, apoptosis, mouse insulinoma cells, cytochrome C/Apaf-1/caspase-3, apoptosis inducing factor pathway

Diabetes mellitus (DM) is a kind of metabolic disease which is characterized by excessively high blood glucose level and classified into two categories, type 1 and type 2 diabetes. There are many Chinese medicines (CM) used to treat DM, including formulae, single Chinese herb and their effective ingredients.[1] Berberine is the major active ingredient isolated from *Rhizoma coptidis*[2] and one of the most promising components which have anti-diabetic effect in CM. Growing evidence demonstrated that berberine has effects of improving obesity and type 2 diabetes mellitus.[3] However, the impact of berberine on pancreatic β-cell is still not fully understood.

Several researchers investigated the effect of berberine on pancreatic β-cell. Zhou, et al[4] found the berberine administration protect against β-cell damage in streptozotocin (STZ) and a high-carbohydrate/high-fat diet induced diabetic rats (150 and 300 mg/kg) as well as in high fructose diet induced insulin resistance rats (187.5 mg/kg for 8 weeks).[5] Berberine has also been reported to increase insulin expression, β-cell regeneration *in vivo*[4] and exerted the insulinotropic effect in rat islets.[6] Further, Xue, et al[7] found that extrapping berberine into solid lipid nanoparticles was more efficient than berberine in promoting islet function

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and protecting the islet from regeneration by morphologic analysis. However, Shen, et al[8] found that berberine (50 mg·kg^{-1}·day^{-1}) treatment significantly reduced the area of islets in high-fat diet fed mice. Berberine (1 μmol/L) inhibited Ins2 mRNA expression and decreased cellular insulin content of pancreatic β-cell line NIT-1 cell.[8] Increasing evidence on the toxicity of berberine showed that the incidence of toxic side effects was related to the doses.[9,10] Thus, the disputes about the toxicity of berberine have not been resolved.[9,10] In the current study, we investigated the pro-apoptotic impact of berberine on the mouse insulinoma (MIN6) cells, which is an important mouse insulinoma cell line.

METHOD

Drugs and Reagents

Berberine was obtained from Zelang Medical Technology Co., Ltd. (Nanjing, China, No. ZL2013083). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was obtained from Sigma-Aldarich (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit and enhanced chemiluminescence (ECL) detection reagent were purchased from Thermo Scientific (Waltham, MA, USA). Specific antibodies against β-actin, AIF, Bax, Bcl-2, caspase-3, and poly adenosine diphosphate-ribose polymerase (PARP) were obtained from Cell Signaling Technology (Boston, MA, USA). Anti-Apaf-1 and anti-cytochrome C antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Annexin V-FITC kit was bought from NeoBioscience Technology Co., Ltd. (Beijing, China). Other reagent-grade chemicals were obtained from commercial sources. MIN6 cells were obtained from Dr. J. Miyazaki (Osaka University, Japan).

Cell Culture

Mouse insulinoma MIN6 cells were cultivated in Dulbecco's modified eagle medium (DMEM) containing 5.5 mmol/L glucose and 10% fetal calf serum (FCS), 100 IU/mL penicillin, 100 μg/mL streptomycin in an incubator at an atmosphere of 5% CO_2 at 37 ℃. The cells were subcultured every 2 or 3 days. All cell culture supplies were purchased from Invitrogen (Carlsbad, CA).

MTT Assay

The viability of MIN6 cells was detected by the MTT reduction conversion assay. Briefly, MIN6 cells were seeded into 96-well plates at a density of 1×10^4 cells per well. After overnight attaching, the cells were incubated with berberine at the concentrations of 1.25, 2.5, 5, 16, 20 and 50 μmol/L separately for 16 h or with 5 μmol/L berberine for various time points (2, 4, 8, 16 and 24 h). After treatment, 10 μL of MTT at a concentration of 5 mg/mL was supplemented into each well, and the whole plate was continuously incubated at 37 ℃ for 4 h. Discarding the untransformed MTT, the crystal products generated from dehydrogenase reduction on MTT substrate in mitochondria was solubilized with 150 mL dimethyl sulfoxide. After shaking the plate for 10-min at room temperature, the absorbance was measured at 450 nm applying EnSpire 2300 multiplate reader (PerkinElmer, USA). The cells were detected in the same way after the treatment of STZ.

DNA Fragmentation Assay

Histone-associated DNA fragmentation in the cytoplasm is associated with cell viability,[11] a special enzyme-linked immuno sorbent assay (ELISA) kit was used to quantify the expected increase of cytoplasmic histone-associated DNA fragments, including mono- and oligo nucleosomes. MIN6 cells were subcultured at a density of 2×10^4 cells/cm^2 and incubated with different concentrations of berberine for 16 h. The presence of mono- and oligonucleosomes in apoptosis was measured by histone-associated DNA fragments in the cytoplasm. The degree of cell apoptosis was determined by cell death detection ELISA plus kit (Roche, cat. No.11774425001).[12] Briefly, cells were collected by being centrifuged at 200×*g* for 10 min and lysed for 30 min with the buffer provided. Aliquots of the supernatants (20 μL) were transferred to streptavidin-coated plates, and then anti-histone-biotin and anti-DNA-peroxidase antibody were supplemented and co-incubated for 2 h, before the 2,2'-azino-bis substrate incubation for 10 min, and then measured as a ratio of absorbance at 405 and 490 nm using Perkin Elmer EnSpire multiplate reader (PerkinElmer, USA). Under the condition of severe cell stress or DNA damage, etc., the corresponding signals were produced to active the intrinsic pathway of apoptosis.[13] Usually, the mitochondrial-associated pathway was considered as the typical one to trigger apoptosis, during which the pro-apoptotic proteins transferred to cytosol to activate caspase proteases from mitochondria are the crucial change. To further discover the mechanism of berberine on MIN6 cell apoptosis, the levels of pro- and anti-apoptotic proteins from whole cell lysate were measured.[14]

Western Blotting

The cells were seeded in 6-well plates and cultivated in DMEM in the presence of various concentrations of

berberine for 16 h. Cells were harvested, washed with cold phosphate buffered saline (PBS) twice and re-suspended in lysis buffer, which contained 20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100, and protease inhibitor cocktail. Protein concentrations were detected by BCA protein assay kit. The 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the nitrocellulose membranes (Bia-Rad, USA) were used for separating and transferring proteins, respectively. And then the membranes were blocked with 5% skim milk in tris-buffered saline and Tween-20 (TBST) buffer for 1 h and incubated overnight at 4 ℃ with rabbit polyclonalanti-AIF, rabbit anti-Apaf-1, rabbit anti-Bax, rabbit anti-Bcl-2, rabbit anti-cleaved caspase-3, rabbit anti-cytochrome C and rabbit anti-PARP antibodies diluted at 1:1000 or anti-β-actin at 1:5000, respectively. Then the membranes were incubated with the appropriate secondary antibodies for 1 h. Subsequently, the signals were detected using a hypersensitive enhanced chemiluminescence reagent and visualized with Alpha Innotech FluorChem 8900 Imager (San Leandro, CA, USA).

Flow Cytometry

Flow cytometry was applied to measure the apoptotic cell after the treatment of berberine by using the Annexin V-FITC kit on the FASC Santo Ⅱ (BD, USA) as previous reported.[15,16] The MIN6 cells was incubated with 5 and 10 μmol/L berberine for 8 and 16 h, respectively.

Statistical Analysis

Data were expressed as mean ± standard error ($\bar{x} \pm SE$). The difference among groups was compared and analyzed by one-way ANOVA using Graphpad software. Values were considered statistically significant when *P* value was less than 0.05. Results were representative of more than three individual experiments.

RESULTS

Berberine Dose-Dependently Decreased MIN6 Cell Viability

Compared with the stretched out untreated cells, berberine-treated MIN6 cells appeared significant cell condensation and cell debris under microscope (Figure 1A). The morphological change was comparable to that caused by overnight incubation with STZ, as reported.[17] MTT assay revealed that berberine dose-dependently decreased cell viability assay, starting from 2.5 up to 50 μmol/L (Figure 1B). The IC_{50} value of berberine on MIN6 cell viability after 16 h treatment was estimated at 5.7 μmol/L. Using 5 μmol/L concentration, we further determined the time course from 2 to 24 h after the addition of berberine. The results revealed that berberine caused a significant 20% reduction in cell number after only 4 h incubation, which reached 50% after 24 h (Figure 1C).

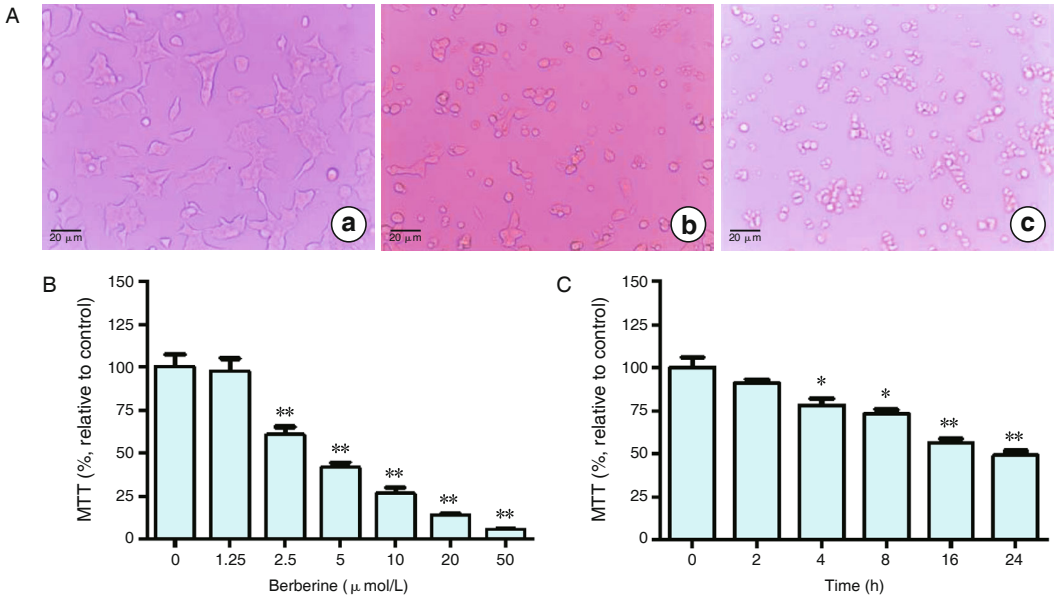

Figure 1. Transient Exposure of Berberine Decreased Cell Viability

Notes: A: morphologic appearance of MIN6 cells after berberine incubation for 16 h (400×); a: control, b: berberine 5 μmol/L; c: berberine 10 μmol/L; representative images were presented from 3 different independent experiments; bar = 20 μm. B : treatment with berberine for 16 h induced a dose-dependent decrease in viability of MIN6 cells detected by MTT assay. C: 5 μmol/L berberine incubation for different time induced decrease in cellular viability. Data were expressed as means ± SE of three independent experiments, n=4; $^{*}P$<0.05, $^{**}P$<0.01, compared with the untreated cells.

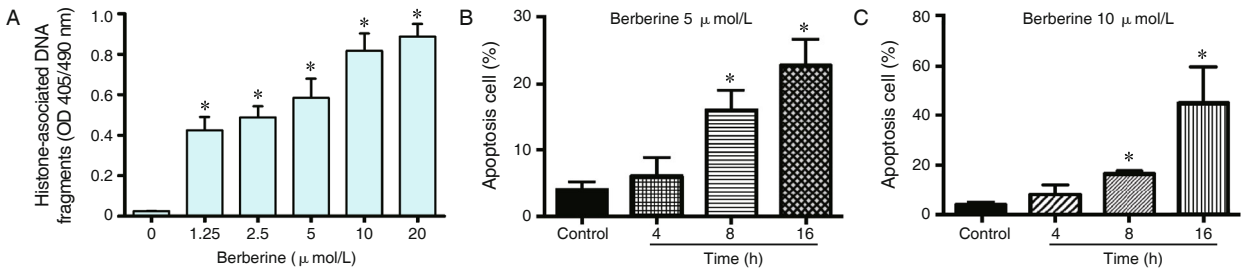

Figure 2. Berberine Dose-Dependently Induce Cell Apoptosis in MIN 6 Cells

Notes: A: changes in histone-associated DNA fragmentation was quantified using ELISA. Data were shown as the means ± SE of three independent experiments; n=5; *P<0.01, compared with the untreated cells. B and C: FACS results showed that apoptotic cells increased 2.9- and 4.6-fold respectively when they were incubated with 5 μmol/L berberine for 8 and 16 h, while counterpart 10 μmol/L increased 3- and 8.7-fold; n=6, *P<0.01, compared with the control group

Berberine Dose-Dependently Induced Cell Apoptosis

As shown in Figure 2A, DNA fragmentation level has a significant increase after treatment with berberine for 16 h in MIN6 cells. The effect was statistically significant at the lowest concentration of 1.25 μmol/L and further increased in a dose-dependent manner up to 20 μmol/L. The increase in DNA fragmentation suggested that berberine could induce cell apoptosis at specific concentrations.

In order to further investigate if berberine could induce cell apoptosis, MIN6 cells were incubated with or without berberine (5 and 10 μmol/L) for certain time, then the cells were harvested with MIN6 and stained with PI and/or annexin-V-FITC. Flow cytometry results showed that the number of apoptotic cells increased about 2.9- and 4.6-fold compared to control after treatment with berberine (5 μmol/L) for 8 and 16 h, while 3 and 8.7-fold after 10 μmol/L treatment for 8 and 16 h, respectively (P<0.01, Figures 2B, 2C). Flow cytometry results further revealed 5 and 10 μ mol/L of berberine may result in cell apoptosis in MIN6 cell.

Apoptotic Effect of Berberine Was Comparable and Synergistic to That of STZ

Effects with berberine on MIN6 cells were compared after 16 h incubation, as shown in Figure 3. Using MTT assay, it was confirmed that a decrease in MIN6 viability in a concentration-dependent way caused by 2.5 and 5 mmol/L STZ, down to 33% of control. Berberine (2.5–10 μmol/L) dose-dependently decreased the relative cell number even lower to 15.5%. The effects were comparable to this established cytotoxin. When they were added together, the effect was clearly synergistic and the relative cell number decreased down to 5.5% of control with 5 mmol/L STZ and 10 μmol/L berberine. Using western blotting, STZ was also found caused similar changes in the expressions of pro- and anti-apoptotic proteins as shown in Figure 4 (data not shown).

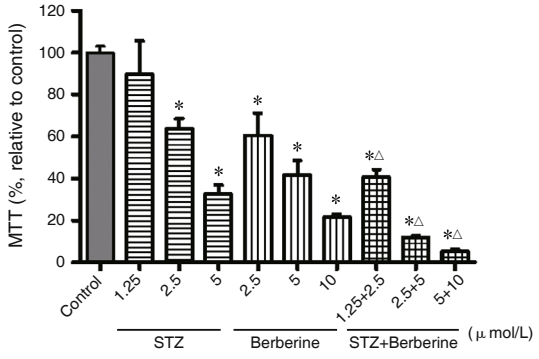

Figure 3. Effect of Berberine on Cell Viability by MTT Assay (n=3)

Notes: *P<0.01, compared with untreated controls; △P<0.05, compared with berberine alone

Berberine Incubation Significantly Increased the Levels of Pro-Apoptotic Proteins

As shown in Figure 4, berberine caused around 35% decrease in the level of anti-apoptotic Bcl-2 and a drastic 23-fold elevation in that of apoptotic Bax proteins. Consequently, a 2.1-fold increase was detected in cytochrome C releasing from mitochondria into the cytoplasm, and 2.3-fold in AIF, followed by a 6.2-fold increase in cellular Apaf-1. The cleavage (into 10- and 17-kDa pieces) and activation of caspase-3 is the final step towards apoptosis. Berberine treatment increased caspase cleavage 2.5-fold. Parp1 activation was an independent parameter of apoptosis, berberine increased Parp1 activation in 2.2-fold.

DISCUSSION

Berberine has been reported to cause apoptosis of human leukemia HL-60, MOLT-4 and K562 cells pancreatic cancer cell lines PANC-1,[18-21] etc. In accordance with these reports, we also found that

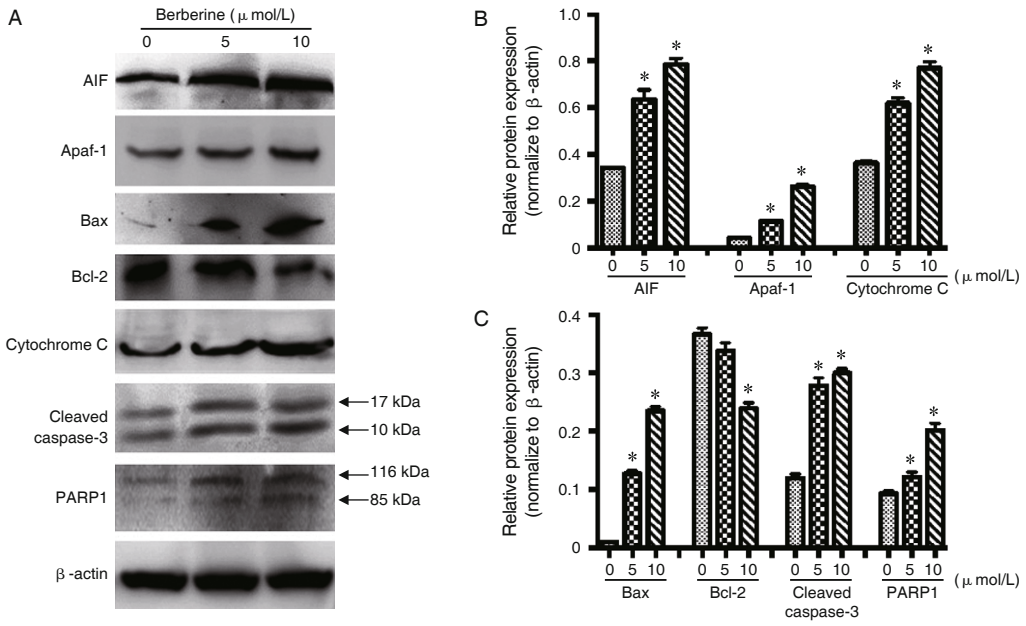

Figure 4. Effect of Berberine on Cell Pro-Apoptotic and Anti-apoptotic Preotein Expression in MIN6 Cells by Western Blotting (*n*=3)

Notes: MIN6 cells were cultured with berberine at the concentration of 5 and 10 μmol/L for 16 h, and the cell lysates were collected and protein expression were analyzed. Representative images of western blots results and densitometric quantification on the protein expression of AIF, Apaf-1, Bax, Bcl-2, cleaved caspase-3, cytochrome C and PARP-1. All the protein expression were normalized to β-actin levels. $^{*}P<0.01$, compared with untreated cells.

a certain concentration of berberine significantly reduced the cell viability and inhibited the cell proliferation in MIN6 cells. These results suggested that improper dosage of berberine may have toxic effects in diabetes treatment. On the other hand, berberine is known to activate AMPK and improve insulin sensitivity, a direct incubation with islet β-cells decreased cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) activity and insulin secretion.[22] To further explore this detrimental effect, we incubated insulinoma MIN6 cells with various concentration of berberine for 16 h.

Previous studies have documented that berberine can acutely decrease fasting insulin secretion and glucose-stimulated insulin secretion in high-fat diet rats and MIN6 cells.[22] However, they also found berberine exerted a toxic effect on MIN6 cells at 2.5 μmol/L or higher for 24 h.[22] In the present study, we found that MIN6 cells gradually demonstrated a series of characters of apoptotic cells as the berberine invention time increases, including anomalous cell morphology, losing of cell mass, broken membrane, cytoplasm vacuoles appearing, condensing chromatin and reducing nucleoli disappeared, and decreasing ratio of nuclear to pulp. Berberine was observed to induce apoptosis in a dose- and time-dependent way in MIN6 cells. Berberine treatment caused a dramatically increasing of DNA fragmentation in MIN6 cells, which confirmed the berberine-induced cellular toxicity. Flow cytometry results also showed that apoptosis was aggravated with the increasing dose and treatment time.

In vivo evidence indicates berberine protected islet β-cells from apoptosis which were induced by STZ, high-fat diet, or lipid toxicity.[5,11,23] Chronic treatment of berberine for 6 weeks in high-fat-diet-fed rats could decrease fasting insulin level and glucose-stimulated insulin secretion. To demonstrate a direct acute effect, berberine with the concentration of 2.5 μmol/L or above for 60 min caused a significant decrease in glucose- and glucose and palmic acid-induced acute insulin secretion. This effect seemed to be mediated by inhibiting cAMP-PKA pathway.[22]

Mitochondria has a crucial effect on instinct pathway of apoptosis, including the release of caspase activators, such as cytochrome C, participation of Bcl-2 family proteins, etc.[23,24] Using anti-apoptotic Bcl-2 and six other pro-apoptotic parameters, we demonstrated that berberine treatment for 16 h caused mitochondria-mediated cell apoptosis. Bax and Bcl-2, two important proteins of Bcl-2

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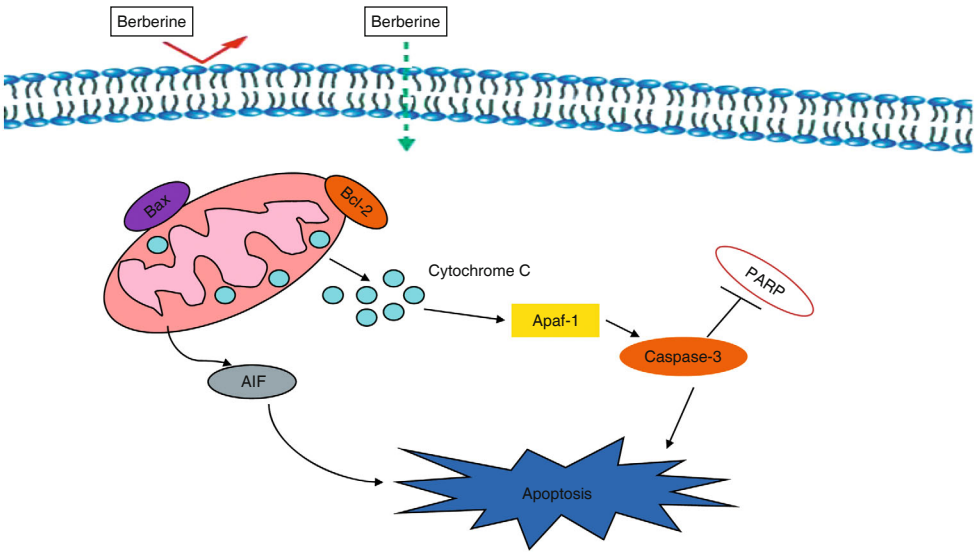

Figure 5. Proposed Mechanism of Berberine-Induced Cell Death in MIN6 Cells

Notes: As a charged molecule, berberine does not cross the cell membrane but may work through cell surface receptors or other proteins. Intracellularly, it causes an increase in Bax but decreases Bcl-2, pro- and anti-apoptotic respectively, which results in increasing release in cytochrome C. Cytochrome C further activates Apaf-1 and activates procasepase-3. The increase expression of cleaved capase-3 further inhibit the expression of poly (ADP-ribose) polymerase1(PARP-1), thus leading to cell apoptosis. On the other hand, AIF causes the nuclear DNA agglutination and breaks into fragments, which also contribute to cell apoptosis

family, are in charge of inducing and protecting from apoptosis. The results of western blotting showed that berberine up-regulated Bax/Bcl-2 protein expression ratio. Berberine does not cross the cell membrane but may work through cell surface receptors or other proteins. Intracellularly, it caused an increase in Bax but decreased Bcl-2, pro- and anti-apoptotic respectively,[25] which results in pathological releasing in cytochrome C. Cytochrome C further activate Apaf-1 and procasepase-3 (Figure 5). Caspase-3 further inhibited the expression poly (ADP-ribose) polymerase1 (PARP-1), thus leading to cell apoptosis. It was reported that PARP-1 induced mitochondrial permeability transition and nuclear translocation of AIF, then triggered cell apoptosis.[26,27] In this study we found significant increase of caspase-3, cytochrome C, Apaf-1 and PARP-1 expression and cleave caspase-3. All these results demonstrated that berberine induced apoptosis in MIN6 cells.

AIF is an important protein exits on the mitochondria inner membrane.[28] It could cause the nuclear DNA agglutination and break into fragments with the size of 50kb when it transfers from mitochondria into the cytoplasm and finally into the nucleus.[29,30] In our study, we found berberine could trigger the expression of AIF, illustrating that berberine-induced MIN6 cell apoptosis may be realized through mitochondrial apoptotic pathway.

On the other hand, AIF causes the nuclear DNA agglutination and break into fragments, which also contributed to cell apoptosis. Our results indicate a transient incubation with berberine (5 μmol/L, 16 h) is sufficient to cause apoptosis of insulin producing cells. This significant side effect will no doubt negatively influence its therapeutic effectiveness against metabolic disorder and diabetes mellitus, especially for its long term or chronic application. What's more, we preliminarily investigated that berberine could decrease the viability of murine primary islet cells in vitro (data not shown).

Although berberine showed cellular toxicity in MIN6 cells, there were few cases that reported the adverse drug reaction after treatment with *Rhizoma coptidis* in clinic. We think the reasons are as follows. Firstly, berberine is an alkaloid extracted from *Rhizoma coptidis*, which only accounts for about 5%–8% of the whole drug.[31] Therefore berberine cannot consider equal to *Rhizoma coptidis*. Secondly, formulae, constructed to treating illnesses in CM usually include many processed Chinese herbs. The toxicity of one herb could be reduced by through processing or matching with others.[32] Lastly, people appeal to drugs when they are suffered from diseases. And in course of diabetes, lots of harmful substances are produced, such as heat, blood stasis and water-dampness. *Rhizoma coptidis* has the function of dispelling dampness and clearing heat in CM, which

is suitable to the unbalanced condition of diabetes. However, the action of the drugs may damage the normal cells when there are not any harmful substance accumulated in cells, which are so called toxic effects.

In summary, berberine (5 and 10 μmol/L) could induce MIN6 cells apoptosis through induction of cytochrome C/Apaf-1/caspase-3 and AIF pathway.

Conflict of Interest

The authors do not have any conflict of interest to declare.

Author Contributions

The conception and study design: Gao SH, Liu JL, Yin HP; acquisition of data, analysis and interpretation of data: Fang X, Miao XL, Mu QQ, Yu N; drafting the article and revising it critically for important intellectual content: Fang X, Miao XL, Zhang DW, Zhao DD, Wang M, Mo FF.

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