Elsevier Editorial System(tm) for Toxicology
Manuscript Draft

Manuscript Number: TOX-11-338

Title: Nickel(II) induced JNK activation-regulated mitochondria-dependent apoptotic pathway leading to cultured rat pancreatic β-cell death

Article Type: Regular Article

Keywords: Nickel (Ni); Pancreatic β-cells; Apoptosis; c-Jun N-terminal kinase (JNK); Mitochondrial dysfunction

Corresponding Author: Associate professor Ya-Wen Chen, Ph. D.

Corresponding Author's Institution: Department of Physiology

First Author: His-Chin Wu, M.D.

Order of Authors: His-Chin Wu, M.D.; Ching-Yao Yang, M.D., Ph.D.; Dong-Zong Hung, M.D., Ph.D.; Chin-Chuan Su, M.D.; Kuo-Liang Chen, M.D.; Cheng-Chien Yen, Ph.D.; Tsung-Jung Ho, M.D., Ph.D.; Yi-Chang Su, M.D., Ph.D.; Chun-Fa Huang, Ph.D.; Chun-Hung Chen, M.D.; Ling-Mei Tsai; Ya-Wen Chen, Ph. D.

Abstract: Nickel (Ni), a well-known toxic metal, is widely used in electroplating and alloy production. It is also an important industrial and environmental pollutant due to uncontrolled industrial and municipal discharges. In this study, we characterize and investigate the cytotoxic effects and its probable toxicological mechanisms of Ni in pancreatic β-cells. The results showed that it was significantly and gradually decreased cell viability after exposure of pancreatic β-cell-derived RIN-m5F cells to NiCl2 for 24 h in a dose-dependent manner. NiCl2 also increased sub-G1 hypodiploid cells and annexin V-Cy3 binding in RIN-m5F cells, indicating that NiCl2 possessed of ability to induce apoptosis. Moreover, exposure of RIN-m5F cells to NiCl2 displayed distinct features of mitochondria-dependent apoptotic signals, including: mitochondria dysfunction (disruption of mitochondrial membrane potential (MMP), increase of mitochondrial cytochrome c release into the cytosol), Bak and Bid mRNA up-regulation, and activation of caspase-3, -7, and -9, and poly(ADP-ribose) polymerase (PARP) degradation. In addition, NiCl2 also markedly induced the activation of c-Jun N-terminal kinases (JNK), but not extracellular signal-regulated kinase (ERK)1/2 and p38. These NiCl2-induced apoptotic-related signaling responses could be effectively reversed by specific JNK inhibitor SP600125. To the best of our knowledge, this study is the first time showing that Ni-caused pancreatic β-cell death through JNK activation-regulated mitochondria-dependent apoptotic signals pathway.

*Cover Letter

Professor K. B. Wallace

Managing Editor for the Americas

Toxicology Editorial Office

Dear Editor:

We are submitting our manuscript entitled "**Nickel(II) induced JNK activation-regulated mitochondria-dependent apoptotic pathway leading to cultured rat pancreatic β-cell death**" to you. The aim of this study is to explore the action mechanisms of nickel(II) induced pancreatic β-cells toxicity, which is widely used in electroplating and alloys producing. Our results in the first time demonstrated that nickel significantly induced rat pancreatic β-cell line (RIN-m5F cells) apoptosis via a JNK activation-regulated mitochondrial-dependent apoptotic pathway and provided evidence that nickel is an important environmental risk factor for β-cell damage.

This paper has not been published and will not be submitted elsewhere for publication. My colleagues and I will like to publish this paper in *Toxicology Letters.*

Your kindness in reviewing our paper would be greater appreciated.

Sincerely yours,

Ya-Wen Chen, Ph.D.
Department of Physiology,
College of Medicine, China Medical University,
No.91 Hsueh-Shih Rd., Taichung 404, Taiwan
Tel: + 886 4 22052121 ext. 7728
Fax: + 886 4 22333641
E-mail: ywc@mail.cmu.edu.tw

To Dr. D. Jones

Administrative Support Agent

Toxicology Editorial Office

Dear Dr. Jones:

We have resubmitted our manuscript entitled "**Nickel(II) induced JNK activation-regulated mitochondria-dependent apoptotic pathway leading to cultured rat pancreatic β-cell death**" to you, again. We were carefully revised according to 'Technical Check Results' valuable suggestions. Please reconsider to publish this manuscript in Toxicology. Further suggestions on our revised manuscript will be greatly appreciated.

With the best wishes,

Sincerely yours,

Ya-Wen Chen, Ph.D.
Department of Physiology,
College of Medicine, China Medical University,
No.91 Hsueh-Shih Rd., Taichung 404, Taiwan
Tel: + 886 4 22052121 ext. 7728
Fax: + 886 4 22333641
E-mail: ywc@mail.cmu.edu.tw

Comments:

Q1. In its current state, the level of English throughout your manuscript does not meet the journal's desired standard. There are a number of grammatical errors and instances of badly worded/constructed sentences. Please check the manuscript and refine the language carefully.

Ans. We have a professional English editor carefully proofread this manuscript, and the errors of English grammar and the worded/constructed sentences have been corrected and modified (mark with red color). If further editorial corrections are needed, we will be pleased to revise according to your suggestions.

Q2. Each bullet point provided in 'Research highlights' should not exceed a maximum of 85 characters including spaces.

Ans. Each bullet point provided in ‘Research highlights’ has been reduced, as follows:

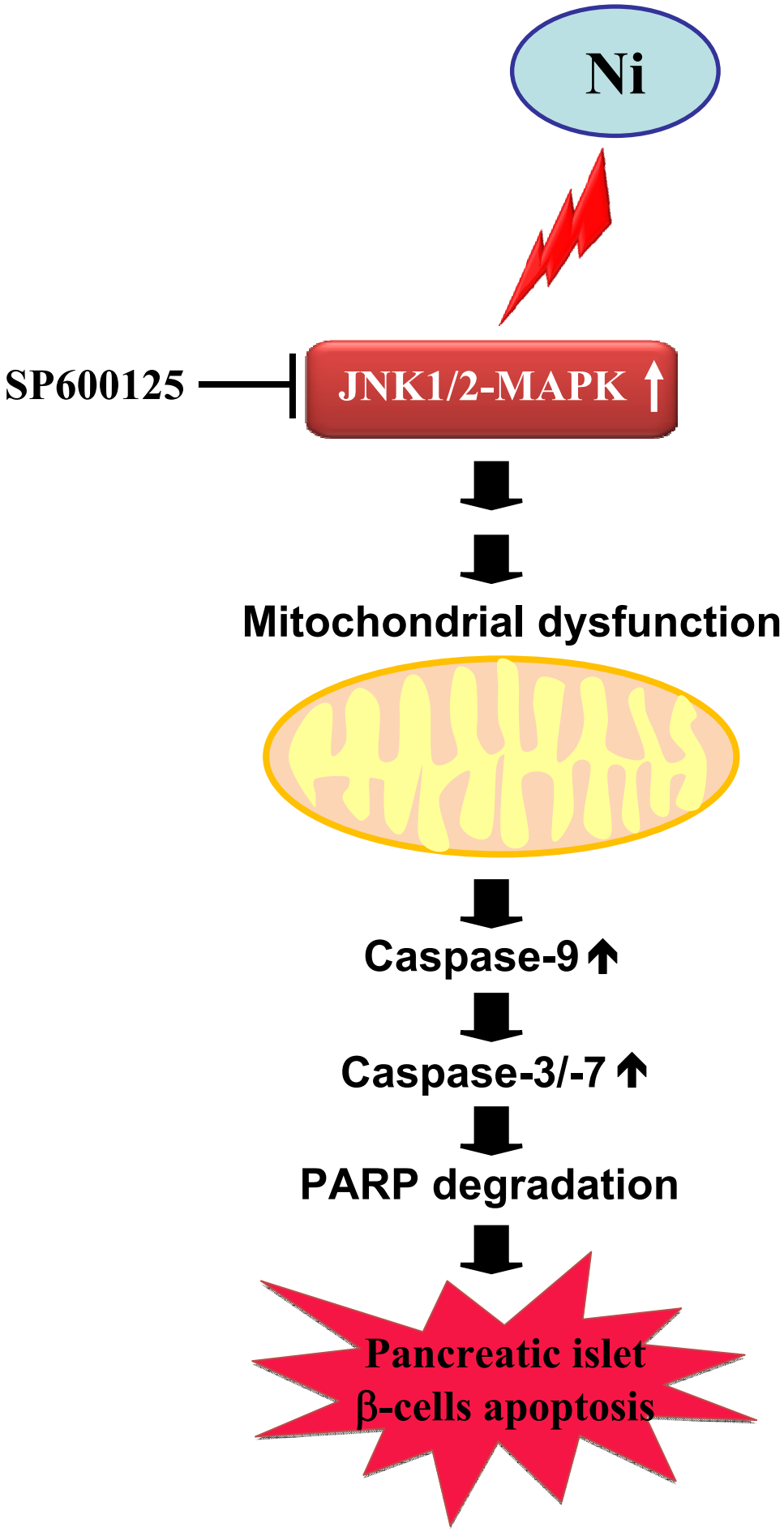
Research Highlights:

►The toxicological effect of nickel (Ni) on the cell death of pancreatic β-cells was examined.

►Ni caused cell death via the mitochondria-dependent apoptotic signals pathway.

►The activation of JNK-MAPK pathway was involved in Ni-induced apoptosis in the pancreatic β-cells.

Graphical Abstract



Research Highlights:

►The toxicological effect of nickel (Ni) on the cell death of pancreatic β-cells was examined.

►Ni caused cell death via the mitochondria-dependent apoptotic signals pathway.

►The activation of JNK-MAPK pathway was involved in Ni-induced apoptosis in the pancreatic β-cells.

*Manuscript
Click here to view linked References

Nickel(II) induced JNK activation-regulated mitochondria-dependent apoptotic pathway leading to cultured rat pancreatic β-cell death

Hsi-Chin Wu[a,1], Ching-Yao Yang[b,1], Dong-Zong Hung[c,1], Chin-Chuan Su[d,1], Kuo-Liang Chen[a], Cheng-Chien Yen[f], Tsung-Jung Ho[g], Yi-Chang Su[g], Chun-Fa Huang[g], Chun-Hung Chen[h], Ling-Mei Tsai[i], Ya-Wen Chen[i,*]

[a]Department of Urology, China Medical University Hospital, No. 2, Yuh-Der Rd., Taichung 404, Taiwan.

[b]Department of Surgery, National Taiwan University Hospital, and Department of Surgery, College of Medicine, National Taiwan University, No. 7, Chung Shan S. Rd., Taipei 100, Taiwan.

[c]Toxicology Center, China Medical University Hospital, No. 2, Yuh-Der Rd., Taichung 404, Taiwan.

[d]Department of Otorhinolaryngology, Head and Neck Surgery, Changhua Christian Hospital, No.135 Nanxiao St. Changhua City, 500 Changhua County, Taiwan.

[f]Department of Occupational Safety and Health, College of Health Care and Management, Chung Shan Medical University; and Department of Occupational Medicine, Chung Shan Medical University Hospital, No. 110, Sec. 1, Jian-Guo N. Rd., Taichung 402, Taiwan.

[g]School of Chinese Medicine, College of Chinese Medicine, China Medical University, No.91 Hsueh-Shih Rd., Taichung 404, Taiwan.

[h]Department of Emergency, China Medical University Hospital, Taichung 404, Taiwan

[i]Department of Physiology and Graduate Institute of Basic Medical Science, College of Medicine, China Medical University, No.91 Hsueh-Shih Rd., Taichung 404,

Taiwan.

To telephone, fax numbers, e-mail address, and postal address of corresponding author:

Tel.: +886 4 22052121 ext.7728

Fax: +886 4 22333641

E-mail: ywc@mail.cmu.edu.tw

Postal address: Department of Physiology, College of Medicine, China Medical University, No.91 Hsueh-Shih Rd., Taichung 404, Taiwan.

Footnotes:

[1]These authors contribute equally to this work.

Abbreviations

MMP, mitochondrial membrane potential; PARP, poly(ADP-ribose) polymerase; JNK, c-Jun N-terminal kinases; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; DM, diabetes mellitus.

Abstract

Nickel (Ni), a well-known toxic metal, is widely used in electroplating and alloy production. It is also significantly implicated in the industrial and environmental pollution due to uncontrolled industrial and municipal discharges. In this study, we characterized and investigated the cytotoxic effects of Ni exposure and their probable toxicological mechanisms in pancreatic β-cells. The results showed that it was significantly decreased cell viability after exposure of pancreatic β-cell-derived RIN-m5F cells to $NiCl_2$ for 24 h, in a dose-dependent manner. $NiCl_2$ also increased sub-G1 hypodiploid cells and annexin V-Cy3 binding population in RIN-m5F cells, indicating its apoptosis-inducing ability. Moreover, exposure of RIN-m5F cells to $NiCl_2$ induced distinct signals of mitochondria-dependent apoptosis, including mitochondrial dysfunction (the disruption of MMP and increase in mitochondrial cytochrome c release into the cytosol), *Bak* and *Bid* mRNA up-regulation, and activation of caspase-3, caspase-7, and caspase-9, and poly(ADP-ribose) polymerase (PARP) degradation. In addition, $NiCl_2$ also markedly induced the activation of c-Jun N-terminal kinases (JNK), but not of extracellular signal-regulated kinase (ERK)1/2 and p38. These $NiCl_2$-induced apoptosis-related signaling responses could be effectively reversed by specific JNK inhibitor SP600125. To the best of our knowledge, this study is the first time to show that Ni causes pancreatic β-cell death through a JNK activation-regulated mitochondria-dependent apoptosis-signals pathway.

Keywords: Nickel (Ni); Pancreatic β-cells; Apoptosis; c-Jun N-terminal kinase (JNK); Mitochondrial dysfunction

1. Introduction

Nickel (Ni), a natural and abundant heavy metal that occurs in igneous rocks in its free form or as iron complexes, is hazardous but, at the same time, recognized as an essential trace element (Yusuf et al., 2011). Ni is a useful metal and has a wide range of industries applications (for example, in electroplating and in production of alloys, jewelry, stainless steel, and electrical batteries) and in orthodontics (such as in dental alloys) (Alloway, 1995; Thompson, 2000). Ni is, however, a severe pollutant to the environment; it has reached concentrations of up to 26,000 ppm in polluted soils and led to human exposure occurrence by its use as a raw material in metallurgical and electroplating works, and through groundwater via the skin or oral uptake (Easton, 1992). Thus, Ni has become an increasingly serious concern for human health over the last few decades. Recent reports have indicated that 32 industrial workers who accidently ingested Ni-contaminated water (containing Ni at levels of 1.63 mg/mL) rapidly developed symptoms of Ni toxicity, including nausea, vomiting, abdominal pain, diarrhea, headache, cough and shortness of breath (ATSDR 2005; HPA 2009; Sunderman et al., 1988). Epidemiological studies have also shown that the occupational workers exposed to Ni-containing compounds (through inhalation, ingestion, or dermal absorption) have suffered serious pathophysiological damage, including pneumonitis, rhinitis, dermatitis, nasal cavity and lung cancer, and even death due to adult respiratory distress syndrome (ARDS) (Cai et al. 2005; Grandjean 1984; Kaspizak et al., 2003; Phillips et al., 2010; Rendall et al., 1994). Moreover, humans may significantly accumulate Ni through the ingestion of Ni-contaminated food, which represents a toxicological hazard for human health (Chashschin et al., 1994; Das et al., 2008; Iglesias et al., 2008).

Type 1 diabetes mellitus (DM, also known as insulin-dependent or juvenile-onset

diabetes) is the pathogeny of pancreatic islet β-cell death or apoptosis, which causes severe insulin deficiency (Hayashi and Faustman 2003). A growing number of studies have indicated that many toxic insults, including high glucose or exposure to heavy metals, can induce the apoptosis of pancreatic islet β-cells, resulting in glucose homeostasis imbalance (Chen et al., 2006; Druwe and Vaillancourt, 2010). Ni has been found to enter cells through various routes, including phagocytosis or divalent metal ion transporters, bound to various biological components, and subsequently altered the cellular morphology and function (Knopfel et al. 2000). These insults of Ni-induced have also been shown the significantly cytotoxic effects in various cell types, including normal kidney cells (Chen et al. 2010a), epithelial cells (Cortijo et al., 2010; Trombetta et al., 2005), testis cells (Doreswamy et al. 2004), lymphocytes (M'Bemba-Meka et al., 2006), and keratinocytes (Little et al. 1996). Furthermore, increasing evidence indicates that the concentrations of 0.1-7.6 mM (approximately 13-975 μg/mL) Ni ion can kill cells by inducing apoptotic processes through various signaling pathways, including: the mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular protein kinase (ERK) pathway, and the Fas/Fas-ligand- and mitochondria-dependent pathways that are involved in cytochrome *c* release and caspase-3 activation (Ahamed et al., 2011; Li et al. 2009a; Kim et al. 2002). Notably, it has been reported that Ni treatment induces a rapid and drastic increase in blood glucose (hyperglycemia) of experimental animals, which is suggested to cause damage to the islet β-cells of pancreas, leading to a decrease in plasma insulin (hypoinsulinemia) (Bwititi and Ashorobi, 1998; Cartana and Arola, 1992; Gupta et al., 2000; Mas et al., 1986). Serdar et al. (2009) have recently indicated that the plasma Ni level is markedly increased in DM patients with impaired fasting glucose levels compared to the healthy people, which may be associated with Ni-induced toxic effects on pancreatic β-cells and the development of DM. However, there is no study

to clarify the relationship between the toxicological effects of Ni-induced and the related mechanisms of pancreatic β-cell death.

Taken together, in the current study, we tried to explore the mechanisms of Ni-induced pancreatic islet β-cell intoxication and apoptosis. To this aim, we sought to investigate the in vitro effects of Ni on cytotoxicity, genomic DNA fragmentation (sub-G1 DNA content), the externalization of phosphatidylserine (PS), mitochondrial dysfunction (MMP depolarization, cytosolic cytochrome *c* release, and pro-apoptotic and anti-apoptotic Bcl-2 family mRNA expressions), and PARP and caspase cascades activation. In addition, JNK activation has been documented to play an important role in many forms of toxicant- or environmental stresses-induced pancreatic islet β-cell death (Cheon et al. 2010; Cowan and Storey, 2003). Thus, we also examined whether exposure of pancreatic islet β-cells to Ni induced the expression of phosphorylation of JNK protein. Moreover, we also investigated the potential protective effects of the specific JNK inhibitor SP600125 on Ni-induced pancreatic islet β-cell damage.

2. Materials and Methods

2.1. Cell culture

RIN-m5F cell is a rat pancreatic β-cell line, showing the production and secretion of insulin. Cells were purchased from American Type Culture Collection (ATCC, CRL-11605, American Type Culture Collection, Manassas, VA, USA), and culture in a humidified chamber with a 5 % CO_2-95 % air mixture at 37 ℃ and maintained in RPIM 1640 medium (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco/Invitrogen) and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin, Gibco/Invitrogen).

2.2. Cell viability assay

Cells were washed twice with PBS and detached with trypsin-EDTA (Gibco/Invitrogen) from 10-cm^2 dishes. Cells were divided and cultured into 24-well plates (2×10^5 cells/well) and then treated with $NiCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. After incubator, the culture medium was removed and replaced with fresh medium containing 30 μL of 2 mg/mL 3-(4,5-dimethyl thiazol-2-yl-)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich). After 4 hours of incubation, the medium was removed and washed twice with PBS. Then, 1 mL dimethyl sulfoxide (Sigma) was added to dissolve the blue formazan crystals, and 150 μl of the well-mixed mixture was transferred into 96-well plates. An enzyme-linked immunosorbent assay (ELISA) microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) was used for absorbance detection at a wavelength of 570 nm.

2.3. Measurement of sub-G1 DNA content

RIN-m5F cells were detached and washed twice with PBS. PBS was removed, and 1 mL cold 70% (v/v) ethanol was added. The cells were stored at 4°C for 24 hours. Then, ethanol was removed and the cells were washed twice with PBS. The cells were stained with propidium iodide (Sigma-Aldrich) at 4°C for 30 minutes in the dark, and were washed with PBS and subjected to FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA), that emitted a fluorescent signal at 525 nm. Each group was acquired more than 10000 individual cells.

2.4. Detection of apoptotic cells

Apoptosis was assessed using Annexin V, a protein that binds to phosphatidylserine (PS) residues which are exposed on the cell surface during the apoptosis (Yen et al., 2007). Cells were treated with or without $NiCl_2$ for 24 h. After treatment, cells were washed twice with PBS (ph 7.4), and incubated with Annexin V-Cy3 (Ann Cy3) and 6-carboxy fluorescein diacetate (6-CFDA) simultaneously (used Annexin V-Cy3^{TM} apoptosis detection kit, Sigma-Aldrich). After labeled at room temperature, the cells were immediately observed by fluorescence microscopy (Axiovert 200, Zeiss, 200x). Ann Cy3 was available for binding to PS, which was observed as red fluorescence. In addition, cell viability could be measured by 6-CFDA, which was hydrolyzed to 6-CF and appears as green fluorescence. Cells in the early stage of apoptosis would be labeled with both Ann Cy3 (red) and 6-CF (green).

2.5. Determination of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was analyzed using the fluorescent probe $DiOC_6$ (Molecular Probes, Inc, Eugene, OR, USA), which was with a positive charge of a mitochondria-specific fluorophore and localized to mitochondria, and the reduced accumulation of $DiOC_6$ reflected the loss of the mitochondrial permeability transition. Briefly, RIN-m5F cells were plated in 12-well culture plate. After treatment with $NiCl_2$ for 24 h, cells were washed twice of PBS and harvested and loaded with 40 nM $DiOC_6$ for 30 minutes in the dark. MMP was analyzed in a FACScan flow cytometer (Becton Dickinson). The sample of each group was collected more than 10,000 individual cells.

2.6. Western blotting analysis

The cellular lysates were prepared and western blotting was performed as previously described (Chen et al., 2010b). In brief, 50 μg protein from each cell lysate was subjected to electrophoresis on 10% (w/v) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were then blocked for 1 hour in PBS and 0.05% Tween 20 (PBST) containing 5% nonfat dry milk. After blocking, the membrane was incubated with mouse- or rabbit- polyclonal antibodies against cytochrome *c*, JNK-1, ERK1/2, p38, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and phospho-JNK1/2, phospho-ERK1/2, phospho-p38, caspase-3, caspase-7, and caspase-9 (Cell signaling Technology Inc., Beverly, MA, UAS) for 1 h. Membranes were then washed with 0.1% PBST and incubated for 45 minutes with goat anti-mouse or anti-rabbit IgG-conjugated horseradish peroxidase (HRP)-conjugated secondary antibodies. The antibody-reactive bands were revealed by enhanced chemiluminescence reagents

(Amersham Biosciences, Sweden) and exposed to radiographic film (Kodak, Rochester, NY, USA).

2.7. Cytosol cytochrome c detection

The cytosol cytochrome *c* detection method was as described previously (Chen et al. 2010b). In brief, cells were treated with $NiCl_2$ at the indicated time (24 h) and then homogenized with a pestle and mortar in 0.4 M mannitol, 25 mM MOPS (pH 7.8), 1 mM EGTA, 8 mM cysteine, and 0.1 % (w/v) bovine serum albumin (Sigma-Aldrich). The cell debris was then removed via centrifugation at 6,000 × *g* for 2 minutes. The supernatant was then removed and recentrifuged at 12,000 × *g* for 15 minutes to pellet the mitochondria. The supernatant (cytosol) was stored for western blotting analysis.

2.8. Real-time quantitative reverse-transcribed polymerase chain reaction (RT-PCR)

Real-time quantitative RT-PCR was performed as described previously (Lu et al. 2010). Cells were treated with $NiCl_2$ for the indicated times, and total RNA was extracted. Total RNA (5 μg) was heated to 90°C for 5 minutes to remove any secondary structures and then rapidly placed on ice. Samples were then reverse-transcribed into cDNA using AMV RTase (Promega, Madison, WI, USA) at 42°C in reaction buffer containing 2.5 mM dNTPs, 40 U/μL RNasin (Promega), 100 nmol random-hexamer primers, 1×RTase buffer, and 30 U AMV RTase in nuclease-free water at a final volume of 20 μL. The mixture was incubated at 42°C for 60 minutes. Samples were then denatured at 95°C for 10 minutes and placed on ice. qPCR used transgene-specific primers for mouse Bak, Bid, and β-actin were

chosen as according to: Bak, forward: 5′-TTTGGCTACCGTCTGGCC-3′ and reverse: 5′-GGCCCAACAGAACCACACC-3′ (Bozec et al., 2004); Bid, forward: 5′-CACGACCGTGAACTTTAT-3′and reverse: 5′-GCTGTTCTCTGGGACC-3′ (Bozec et al., 2004); β-actin, 5′-TGTGATGGTGGGAATGGGTCAG-3′ and reverse : 5′-TTTGATGTCACGCACGATTTCC-3′ (Min et al., 2009). Each sample was detected using real-time Sybr Green PCR reagent (Invitrogen) with specific primer in a 25-μl reaction volume, and amplification was performed using an ABI Prism 7900HT real-time thermal cycler (Applied Biosystems, Carlsbad, CA, USA). Cycling conditions were 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 92°C for 30 seconds, and 1 minute at 60°C. Real-time fluorescence detection was performed during the 60°C annealing/extension step of each cycle. Melt-curve analysis was performed on each primer set to ensure that no primer dimers or nonspecific amplification was present under the optimized cycling conditions. The fold difference in mRNA expression was determined using the relative quantification method utilizing real-time PCR efficiencies and normalized to the *β-actin* gene, thus comparing relative C_T changes between control and experimental samples. Before conducting statistical analyses, we calculated the fold change from the mean of the control group for each individual sample, including individual control samples, to assess variability within the group.

2.9. Statistical analysis

Data are presented as means ± standard deviation (S.D.). Statistical significance was evaluated by the Student's *t*-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance

(ANOVA), and the Duncans's post hoc test was applied to identify group differences. The *P* value less than 0.05 was considered to be significant. The statistical package SPSS, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

3. Results

3.1. $NiCl_2$ decreases cell viability and induces apoptosis in rat pancreatic β-cell-derived RIN-m5F cells

To examine $NiCl_2$-induced pancreatic islet β-cell cytotoxicity, we first investigated the effect of $NiCl_2$ on cell survival in pancreatic islet β-cell-derived cell line (RIN-m5F cells). Treatment of the cells for 24 h with different concentrations of $NiCl_2$, ranging from 65-325 μg/mL, lead to a significant reduction in the viable cells by using MTT assays, and the LD_{50} was determined to be approximately 181 μg/mL (Figure 1). Next, in order to determine the cytotoxicity of $NiCl_2$-induced in RIN-m5F cells from the point of view of apoptosis, we analyzed the sub-G1 hypodiploid cell population by flow cytometry. Compared to the vehicle-treated RIN-m5F cells, the number of sub-G1 hypodiploid cells was markedly increased in the cells treated with $NiCl_2$ (130 and 195 μg/mL) for 24 h (Figure 2A). We next evaluated annexin V-Cy3/6-CFDA dual-staining as a method for detecting of phosphatudylserine (PS) externalization (a hallmark of early events in apoptosis) (Yen et al., 2007). As shown in Figure 2B, cells treated with $NiCl_2$ (130 and 195 μg/mL) for 24 h were significantly labeled with both Ann-Cy3 (red) and 6-CF (green) fluorescence, which identified them as being in the stage of apoptosis. These results indicate that exposure of pancreatic islet β-cells to $NiCl_2$ can induce apoptosis.

3.2. $NiCl_2$ induces apoptosis via the mitochondria-dependent pathway in RIN-m5F cells

To investigate whether $NiCl_2$-induced apoptosis was mediated through a mitochondria-dependent pathway, we first measured MMP by using flow cytometry

with a cationic dye, $DiOC_6$ to show that $NiCl_2$ affected the mitochondrial permeability transition (Chen et al., 2010b). As show in Figure 3A, the treatment of RIN-m5F cells with $NiCl_2$ (130 and 195 μg/mL) for 24 was triggered the significant decrease in MMP with a dose-dependent manner. We next examined whether MMP depolarization could promote cytochrome *c* release from the mitochondria into the cytosol in $NiCl_2$-treated RIN-m5F cells by western blot analysis. Treatment of cells with $NiCl_2$ for 24 h effectively increased the cytochrome c level in the cytosolic fraction (130 μg/mL $NiCl_2$, 1.31 ± 0.1; 195 μg/mL $NiCl_2$, 1.61 ± 0.11 fold of the control, $n = 6$, $*p < 0.05$)(Figure 3B). Meanwhile, we also determined the change in the expression of pro-apoptotic and anti-apoptotic mRNA following $NiCl_2$ exposure. As shown in Figure 3C, there was markedly induced an increase in the mRNA expression of *Bak* and *Bid* (pro-apoptotic) after exposure of the cells to $NiCl_2$ (130 and 195 μg/mL) for 24 h. However, the mRNA expression of *Bcl-2* (anti-apoptotic) was not altered (data not shown). Therefore, $NiCl_2$ could induce a significant shift in the pro-apoptotic/anti-apoptotic Bcl-2 expression ratio, which led the cells toward an apoptosis-associated state.

Furthermore, the cleaved forms of PARP and the activation of caspase cascades (one of the critical biomarkers of apoptosis) were also detected. As shown in Figure 4A, the level of the active form of PARP (the 89-kDa cleaved fragment) was significantly increased after treatment of RIN-m5F cells with $NiCl_2$ (130 and 195 μg/mL) for 24 h. It was also observed the remarkable activation of caspase-3, caspase-7, and upstream caspase-9 in $NiCl_2$-treated cells (Figures 4B, 4C, and 4D).

3.3. JNK-MAPK signal pathway activation was the upstream effector of $NiCl_2$-induced apoptosis in RIN-m5F cells.

It has been reported that MAPKs play an important role in the apoptosis

signaling pathway (Cowan and Storey, 2003). However, the possible role of MAPKs in $NiCl_2$-induced pancreatic islet β-cell apoptosis was unclear. Therefore, MAPKs activations (phosphorylation) were investigated. As shown in Figure 5A, the phosphorylation of JNK1/2 protein, but not of ERK1/2 and p38, significantly increased within 30 mins in the $NiCl_2$(130 and 195 μg/mL)-treated RIN-m5F cells compared to the control cells. The effect of $NiCl_2$ on the JNK1/2 pathway could be abolished by pre-treatment of the cells with the specific JNK inhibitor SP600125 (10 μM) (Figure 5B). In addition, $NiCl_2$-induced apoptosis signaling events, including labeling with both Ann-Cy3 (red) and 6-CF (green) fluorescence, the depolarization of MMP, the increase of cytosolic cytochrome c release and pro-apoptotic mRNA expressions, and PARP and caspase cascades activation, were effectively reversed by pre-treatment with SP600125 (Figures 2B, 3, and 4). The phosphorylation of JNK1/2 MAPK was correlated with the induction of apoptosis signaling pathways by $NiCl_2$. Therefore, these results imply that $NiCl_2$ can induce pancreatic islet β-cell apoptosis by regulating the JNK MAPK pathway.

4. Discussion

Nickel (Ni) is widely used in variety of industrial and metallurgical processes, such as electroplating and alloy production, as well as in Ni-cadmium batteries (Alloway, 1995; Easton, 1992; Thompson, 2000). Due to uncontrolled industrial and municipal discharges (the main source of Ni release), the soil and rivers in India and other countries are becoming highly polluted with Ni, with concentrations generally ranging from 35 to 211 mg/L (ppm), even reaching above 70,900-511,000 mg/L in the sediments of contaminated rivers (Alloway, 1995; Igwilo et al., 2006; Israili, 1992). Thus, Ni is a severe hazard for human health. Epidemiological studies have shown that exposure to Ni, which can accumulate in mammals, induces cellular or organ damage and many other pathogenic processes (Das et al., 2008; Grandjean 1984; Kaspizak et al., 2003; Phillips et al., 2010). It has also been indicated that Ni can cause many types of cell death by inducing apoptosis-signaling pathways. In these reports, the doses of Ni resulting in cytotoxic effects ranged from 0.1 to 7.6 mM (approximately 13 to 975 μg/mL) (Kim et al., 2002; Li et al., 2009b; M'Bemba-Meka et al. 2006; Trombetta et al. 2005). Specifically, some animal studies have indicated that it can induce the marked increase in blood glucose levels after exposure to Ni, which is suggested to damage the pancreatic β-cells leading to lower plasma insulin levels and β-cell dysfunction/death (wititi and Ashorobi, 1998; Cartana and Arola, 1992; Gupta et al., 2000). Moreover, recent reports have indicated that the concentrations of some toxic metals (such as Ni, Pb, As, and Al) are markedly high in the blood and urine sample of DM patients. Similarly, the level of Ni was also significantly high in the plasma of DM patients with impaired fasting glucose compared to the healthy people. Thus, it is suggested that Ni may play a critical role

in the development and progression of DM (Afridi et al., 2008; Serdar et al., 2009). Despite some studies showing that Ni can induce toxicological damage to several kinds of organs or cells, the precise mechanisms underlying the pancreatic β-cell death caused by Ni-exposure are mostly unclear. Thus, to elucidate the effects and signaling mechanisms of Ni-induced cell death in the pancreatic islet β-cells, we examined the in vitro toxicological effects of Ni in pancreatic β-cell-derived RIN-m5F cells. The main findings of this study were revealed that Ni induced RIN-m5F cell cytotoxicity as well as the activation of apoptotic cascades. One of the mechanisms underlying Ni-directed β-cell apoptosis was mitochondrial dysfunction and JNK-MAPK activation. These Ni-induced cytotoxic responses could be reversed by treatment with specific JNK inhibitor SP600125. Therefore, these findings indicate that JNK activation regulated mitochondria-dependent apoptotic signals are involved in Ni-induced pancreatic islet β-cell death.

Apoptosis is associated with changes in the biogenesis and function of mitochondria. Previous studies have reported that extracellular stimuli induce apoptosis through the loss of MMP, which is believed to be associated with cytochrome *c* release from the mitochondria into the cytosol. Cytochrome *c* binds to apoptotic protease activating factor 1, activates procaspase-2 and procaspase-9, and then the activation of caspase-3, caspase-6, and caspase-7, and leads to apoptosis (Kroemer et al. 1997; Raff 1998). Mitochondria are very sensitive to toxic insults, which cause mitochondrial DNA mutation and the disturbances to mitochondrial function. It has been demonstrated that mitochondrial dysfunction plays an important role in mammalian cell apoptosis, and is implicated in the pathogenic processes of many diseases, including DM (Kang and Hamasaki, 2005; Lee et al., 2009). Recent studies have documented that toxic metals (mercury and inorganic arsenic) induce β-cell apoptosis by disrupting mitochondrial function (depolarization of MMP and

cytochrome c release from the intermembrane space of mitochondria to the cytosol) and activating caspase cascade signals (Chen et al., 2006 and 2010b; Druwe and Vaillancourt, 2010). Furthermore, the Bcl-2 (B-cell leukemia/lymphoma 2) family is defined by homology shared within 4 conserved Bcl-2 homology domains (BH1-4), which have been found to be involved in the regulation of apoptosis in the mitochondria (Huang and Strasser, 2000; Shimizu et al., 1999). Many studies have classified the Bcl-2 family into three subfamilies: anti-apoptotic, pro-apoptotic, and BH3 domain-only members. The anti-apoptotic members (consisting of Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bcl2a1/A1 and, presumably, Bcl2l10/Diva/Boo/Bcl-B) display sequence conservation through BH1-4; Bax, Bak, and Bok/Mtd are the pro-apoptotic members, which are further divided into more fully conserved "multidomain" members possessing homology in BH1-3; BH3 domain-only members share sequence homology only in the BH3 domain, and include Bad, Bid, Blk/Biklk, Hrk/DP5, Bim, BNip3, BNip3L/Nix, Noxa, Puma/Bbc3 and Bmf. (Kim et al. 2006; Tsujimoto and Shimizu 2000). A previous study demonstrated that depleted Bax and Bak proved resistant to the intrinsic death signals tested (Wei et al. 2001). Other studies have shown that Bid and Bim play important roles in the initiation of apoptotic cell death (Ghiotto et al. 2010; Huang and Strasser, 2000). In this study, the results showed that Ni was capable of triggering RIN-m5F cell apoptosis by inducing MMP depolarization and increasing cytochrome *c* release. Meanwhile, it was also accompanied with a marked increase in the activation of caspase-9, caspase-7, and caspase-3 proteases and in the levels of the cleaved fragment (active form) of PARP. Moreover, exposure of RIN-m5F cells to Ni resulted in the significant increase in *Bak* and *Bid* mRNA expressions, but not altered *Bcl-2* expression. These results implicate that Ni induces pancreatic islet β-cell death through a mitochondria-dependent apoptosis pathway.

Mitogen-activated protein kinases (MAPKs), including JNK, ERK1/2, and p38, are a family of serine/threonine protein kinases that play a critical role in the regulation of many important cellular functions such proliferation, differentiation, response to environmental stimuli, and apoptosis (Cowan and Storey, 2003; Pearson et al., 2001). Previous studies have reported that the JNK pathway is strongly activated by toxic insults or environmental stresses, which leads to apoptosis (Chen et al., 2010c; Kim et al. 2008)). It has also been indicated that the activation of JNK pathway, which is induced by free fatty acids, is involved in the progression of β-cell death (Yuan et al., 2010). However, until now, there is no study to explore the crucial role of JNK pathway activation in Ni-induced apoptotic signaling cascades with respect to pancreatic islet β-cell death. The present work revealed that exposure of RIN-m5F cells to Ni significantly enhanced the phosphorylation of JNK1/2, but not that of ERK1/2 and p38. The pharmacological JNK inhibitor SP600125 effectively suppressed Ni-induced JNK activation and attenuated apoptosis-related signaling cascades (including the sub-G1 hypodiploid cell population, phosphatidylserine (PS) externalized positive cells, the loss of MMP, cytochrome c release, and the activation of PARP, caspase-3, caspase-7, and caspase-9). These results suggest that Ni-triggered JNK pathway activation regulated apoptotic signal is involved in Ni-induced pancreatic islet β-cell death.

Diabetes Mellitus (DM), a group of metabolic diseases with hyperglycemia caused by defecting in the insulin secretion of pancreatic islet β-cells and/or insulin action on the peripheral tissues, is a global health problem. The crucial feature of type 1 DM (insulin-dependent DM, IDDM) is a severe insulin deficiency, which is caused by an autoimmune disorder that is genetically mediated. Recent studies of type 1 DM have shown that environmental factors (such as viral infections, pollution, and exposure to toxic chemicals such as mercury and cadmium) can induce pancreatic

β-cell disruption or death, and potentially be involved in the pathogenesis of type 1 DM (Chen et al., 2006 and 2010b; Placzkiewicz-Jankowska et al., 2007). On the other hand, Ni has been shown to be a powerful toxic metal that produces damage to β-cells in the pancreas and diabetic complications (Horak and Sunderman, 1975). Studies have reported toxicological effects in experimental animals exposed to Ni, including markedly increased blood plasma glucose levels, which were accompanied with hypoinsulinemia conduction (Bwititi and Ashorobi, 1998; Cartana and Arola, 1992; Chen et al. 2009 Mas et al., 1986). It has also been indicated that the significant increase in the levels of constitutive form of nitric oxide synthase (c-NOS) and cyclic guanosine monophosphate (cGMP) in the adrenal gland and brain, and inducible NOS (i-NOS) in the pancreas, are involved in Ni-induced hyperglycemia, suggesting that nitric oxide stress may contribute to Ni-induced pancreatic β-cell apoptosis (Gupta et al. 2000; Li et al., 2009a). Nevertheless, the cytotoxic effects and toxicological mechanisms of Ni in pancreatic islet β-cell apoptosis are still unclear. In the present study, our observations revealed that Ni significantly decreased cell viability in the pancreatic islet β-cell line (RIN-m5F cells) for 24 h. Treatment with Ni initiated mitochondrial dysfunction and apoptotic cascades, and activated the phosphorylation of JNK1/2, which could be reversed by specific JNK inhibitor SP600125. These results indicate that Ni is capable of inducing pancreatic islet β-cell death through JNK activation regulated mitochondria-dependent apoptosis.

5. Conclusion

Collectively, from the in vitro study performed, our results primarily demonstrate that Ni can induce pancreatic β-cell death by JNK activation downstream leading to mitochondrial dysfunction, which subsequently triggers the activation of caspase cascades and PARP degradation, resulting in apoptosis (Figure 6). Based on the aforementioned observations, we provide evidence to confirm the possibility that Ni may an important environmental risk factor in the progression of pancreatic β-cell damage and in the development of DM.

Conflict of interest

All authors declare that there are no conflicts of interest in this study.

Acknowledgements

This work was supported by research grants form the National Science Council of Taiwan (NSC 98-2314-B-039-015 and NSC 98-2320-B-039-014-MY3), the National Health Research Institutes of Taiwan (NHRI-EX98-974SI and NHRI-EX99-9744SI), and the China Medical University, Taichung, Taiwan (CMU-99-TC-27). It was also supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004).

References

Afridi, H.I., Kazi, T.G., Kazi, N., Jamali, M.K., Arain, M.B., Jalbani, N., Baig, J.A., Sarfraz, R.A., 2008. Evaluation of status of toxic metals in biological samples of diabetes mellitus patients. Diabetes Res. Clin. Pract. 80, 280-8.

Agency for Toxic Substances and Disease Registry (ATSDR), 2005. Toxicological Profile for Nickel. US Department of Health and Human Services. Atlanta, US.

Ahamed, M., Akhtar, M.J., Siddiqui, M.A., Ahmad, J., Musarrat, J., Al-Khedhairy, A.A., Alsalhi, M.S., Alrokayan, S.A., 2011. Oxidative stress mediated apoptosis induced by nickel ferrite nanoparticles in cultured A549 cells. Toxicology (In press).

Alloway, B.J., 1995. In: Alloway BJ (ed) Heavy metal in soils, in Blackie Academic and Professional, London, UK, pp 25-34.

Bozec, A., Chuzel, F., Chater, S., Paulin, C., Bars, R., Benahmed, M., Mauduit, C., 2004. The mitochondrial-dependent pathway is chronically affected in testicular germ cell death in adult rats exposed in utero to anti-androgens. J. Endocrinol. 183, 79-90.

Bwititi, P.T., Ashorobi, R.B., 1998. Effects of chronic oral nickel chloride administration on glycaemia and renal function in normal and diabetic rats. Afr. J. Health Sci. 5, 198-201.

Cai, H.R., Cao, M., Meng, F.Q., Wei, J.Y., Hou, J., 2005. Giant cell interstitial pneumonia in a nickel metal hydride battery worker: a case report and literature review. Chin. Med. J. (Engl) 118, 1668-1672.

Cartana, J., Arola, L., 1992. Nickel-induced hyperglycaemia: the role of insulin and glucagon. Toxicology 71, 181-192.

Chen, C.Y., Lin, T.K., Chang, Y.C., Wang, Y.F., Shyu, H.W., Lin, K.H., Chou, M.C., 2010a. Nickel(II)-induced oxidative stress, apoptosis, G2/M arrest, and genotoxicity in normal rat kidney cells. J. Toxicol. Environ. Health A 73, 529-539.

Chen, Y.W., Huang, C.F., Yang, C.Y., Yen, C.C., Tsai, K.S., Liu, S.H., 2010b. Inorganic mercury causes pancreatic beta-cell death via the oxidative stress-induced apoptotic and necrotic pathways. Toxicol. Appl. Pharmacol. 243, 323-331.

Chen, Y.W., Chen, K.L., Chen, C.H., Wu, H.C., Su, C.C., Wu, C.C., Way, T.D., Hung, D.Z,, Yen, C.C., Yang. Y.T., Lu, T.H., 2010). Pyrrolidine dithiocarbamate (PDTC)/Cu complex induces lung epithelial cell apoptosis through mitochondria and ER-stress pathways. Toxicol. Lett. 199, 333-340.

Chen, Y.W., Yang, C.Y., Huang, C.F., Hung, D.Z., Leung, Y.M., Liu, S.H., 2009. Heavy metals and islet function and diabetes development. Islets 1, 1-8.

Chen, Y.W., Huang, C.F., Tsai, K.S., Yang, R.S., Yen, C.C., Yang, C.Y., Lin-Shiau, S.Y., Liu, S.H., 2006. Methylmercury induces pancreatic beta-cell apoptosis and dysfunction. Chem. Res. Toxicol. 19, 1080-1085.

Cheon, H., Cho, J.M., Kim, S., Baek, S.H., Lee, M.K., Kim, K.W., Yu, S.W., Solinas, G., Kim, S.S., Lee, M.S., 2010. Role of JNK activation in pancreatic beta-cell death by streptozotocin. Mol. Cell. Endocrinol. 321, 131-137.

Cortijo, J., Milara, J., Mata, M., Donet, E., Gavara, N., Peel, S.E., Hall, I.P., Morcillo, E.J., 2010. Nickel induces intracellular calcium mobilization and pathophysiological responses in human cultured airway epithelial cells. Chem. Biol. Interact. 183, 25-33.

Cowan, K.J., Storey, K.B., 2003. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. J. Exp. Biol.

206, 1107-1115.

Chashschin, V.P., Artunina, G.P., Norseth, T., 1994. Congenital defects, abortion and other health effects in nickel refinery workers. Sci, Total Environ. 148, 287-291.

Das, K.K., Das, S.N., Dhundasi, S.A., 2008. Nickel, its adverse health effects & oxidative stress. Indian J. Med. Res. 128, 412-425.

Doreswamy, K., Shrilatha, B., Rajeshkumar, T., Muralidhara, 2004. Nickel-induced oxidative stress in testis of mice: evidence of DNA damage and genotoxic effects. J. Androl. 25, 996-1003.

Druwe, I.L., Vaillancourt, R.R., 2010. Influence of arsenate and arsenite on signal transduction pathways: an update. Arch. Toxicol. 84, 585-596.

Easton, D.F., 1992. Nickel and human health: current perspectives, in: Nieboer, E., Nriagu, J.O. (Eds.), Wiley, New York, pp. 603-619.

Ghiotto, F., Fais, F., Bruno, S., 2010. BH3-only proteins: the death-puppeteer's wires. Cytometry A 77, 11-12.

Grandjean, P., 1984. Human exposure to nickel. IARC Sci. Publ. 469-485.

Gupta, S., Ahmad, N., Husain, M.M., Srivastava, R.C., 2000. Involvement of nitric oxide in nickel-induced hyperglycemia in rats. Nitric Oxide 4, 129-138.

Hayashi, T., Faustman, D.L., 2003. Role of defective apoptosis in type 1 diabetes and other autoimmune diseases. Recent. Prog. Horm. Res. 58, 131-153.

Health Protection Agency (HPA), 2009. Nickel, Toxicological overview. UK

Horak, E., Sunderman, F.W. Jr., 1975. Effects of Ni(II) upon plasma glucagon and glucose in rats. Toxicol. Appl. Pharmacol. 33, 388-391.

Huang, D.C., Strasser, A., 2000. BH3-Only proteins-essential initiators of apoptotic cell death. Cell 103, 839-842.

Iglesias, A.V., Burgos, D.S., Marchetti, P.N., Silva, Z.C., Pino, Z.P., 2008. [Urinary nickel in children exposed to petcoke pollution]. Rev, Med, Chil, 136,

1039-1046.

Igwilo, I.O., Afonne, O.J., Maduabuchi, U.J., Orisakwe, O.E., 2006. Toxicological study of the Anam River in Otuocha, Anambra State, Nigeria. Arch, Environ, Occup, Health 61, 205-208.

Israili, A.W., 1992. Occurrence of heavy metals in Ganga river and sediments. Indian J. Environ. Health 34, 63-66.

Kang, D., Hamasaki, N., 2005. Alterations of mitochondrial DNA in common diseases and disease states: aging, neurodegeneration, heart failure, diabetes, and cancer. Curr. Med. Chem. 12, 429-441.

Kasprzak, K.S., Sunderman, F.W. Jr., Salnikow, K., 2003. Nickel carcinogenesis. Mutat. Res. 533, 67-97.

Kim, H., Rafiuddin-Shah, M., Tu, H.C., Jeffers, J.R., Zambetti, G,P,, Hsieh, J.J., Cheng, E.H., 2006. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat. Cell. Biol. 8, 1348-1358.

Kim, K., Lee, S.H., Seo, Y.R., Perkins, S.N., Kasprzak, K.S., 2002. Nickel(II)-induced apoptosis in murine T cell hybridoma cells is associated with increased fas ligand expression. Toxicol. Appl. Pharmacol. 185, 41-47.

Kim, J.Y., Huh, K.H., Park, Y.J., Fang, Y., Kang, C.M., Kim, Y.S., 2008. Molecular mechanisms of cell death of mycophenolic acid-treated primary isolated rat islets: implication of mitogen-activated protein kinase activation. Transplant. Proc. 40, 2575-2577.

Knopfel, M., Schulthess, G., Funk, F., Hauser, H., 2000. Characterization of an integral protein of the brush border membrane mediating the transport of divalent metal ions. Biophys. J. 79, 874-884.

Kroemer, G., Zamzami, N., Susin, S.A., 1997. Mitochondrial control of apoptosis. Immunol. Today 18, 44-51.

Lee, J.W., Kim, W.H., Lim, J.H., Song, E.H., Song, J., Choi, K.Y., Jung, M.H., 2009. Mitochondrial dysfunction: glucokinase downregulation lowers interaction of glucokinase with mitochondria, resulting in apoptosis of pancreatic beta-cells. Cell Signal. 21, 69-78.

Li, B.F., Liu, Y.F., Cheng, Y., Zhang, J.L., Wang, B.G., 2009a. The effect of iNOS gene expression inhibited by RNA inference on the pancreas islet apoptosis and function in rats. Zhonghua Wai Ke Za Zhi 47, 1406-1409.

Li, Q., Suen, T.C., Sun, H., Arita, A., Costa, M., 2009b. Nickel compounds induce apoptosis in human bronchial epithelial Beas-2B cells by activation of c-Myc through ERK pathway. Toxicol. Appl. Pharmacol. 235, 191-198.

Little, M.C., Gawkrodger, D.J., MacNeil, S., 1996. Chromium- and nickel-induced cytotoxicity in normal and transformed human keratinocytes: an investigation of pharmacological approaches to the prevention of Cr (VI)-induced cytotoxicity. Br. J. Dermatol. 134, 199-207.

Lu, T.H., Chen, C.H., Lee, M.J., Ho, T.J., Leung, Y.M., Hung, D.Z., Yen, C.C., He, T.Y., Chen, Y.W., 2010. Methylmercury chloride induces alveolar type II epithelial cell damage through an oxidative stress-related mitochondrial cell death pathway. Toxicol. Lett. 194, 70-78.

Mas, A., Alemany, M., Arola, L., 1986. Effects of a nickel load upon the concentration of plasma metabolites in pregnant rats. Gynecol. Obstet. Invest. 21, 193-197.

M'Bemba-Meka, P., Lemieux, N., Chakrabarti, S.K., 2006. Role of oxidative stress, mitochondrial membrane potential, and calcium homeostasis in human lymphocyte death induced by nickel carbonate hydroxide in vitro. Arch. Toxicol. 80, 405-420.

Min, H.Y., Kim, M.S., Jang, D.S., Park, E.J., Seo, E.K., Lee, S.K., 2009. Suppression of lipopolysaccharide-stimulated inducible nitric oxide synthase (iNOS)

expression by a novel humulene derivative in macrophage cells. Int. Immunopharmacol. 9, 844-9.

Pearson, G., Robinson, F., Beers, Gibson, T., Xu, B.E., Karandikar, M., Berman, K., Cobb, M.H., 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev. 22, 153-183.

Phillips, J.I., Green, F.Y., Davies, J.C., Murray, J., 2010. Pulmonary and systemic toxicity following exposure to nickel nanoparticles. Am. J. Ind. Med. 53, 763-767.

Placzkiewicz-Jankowska, E., Szybinski, Z., Huszno, B., 2007. [Environmental factors in the development of type 1 diabetes -- a new insight]. Przegl. Lek. 64, 175-179.

Raff, M., 1998. Cell suicide for beginners. Nature 396, 119-122.

Rendall, R.E., Phillips, J.I., Renton, K.A., 1994. Death following exposure to fine particulate nickel from a metal arc process. Ann. Occup. Hyg. 38, 921-930.

Serdar, M.A., Bakir, F., Hasimi, A., Celik, T., Akin, O., Kenar, L., Aykut, O., Yildirimkaya, M., 2009. Trace and toxic element patterns in nonsmoker patients with noninsulin-dependent diabetes mellitus, impaired glucose tolerance, and fasting glucose. Int. J. Diabetes Dev. Ctries. 29, 35-40.

Shimizu, S., Narita, M., Tsujimoto, Y., 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399, 483-487.

Sunderman, F.W. Jr., Dingle, B., Hopfer, S.M., Swift, T., 1988. Acute nickel toxicity in electroplating workers who accidently ingested a solution of nickel sulfate and nickel chloride. Am. J. Ind. Med. 14, 257-266.

Thompson, S.A., 2000. An overview of nickel-titanium alloys used in dentistry. Int Endod. J. 33, 297-310.

Trombetta, D., Mondello, M.R., Cimino, F., Cristani, M., Pergolizzi, S., Saija, A.,

2005. Toxic effect of nickel in an in vitro model of human oral epithelium. Toxicol. Lett. 159, 219-225.

Tsujimoto, Y., Shimizu, S., 2000. Bcl-2 family: life-or-death switch. FEBS Lett. 466, 6-10.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., Korsmeyer, S.J., 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292, 727-730.

Yen, C.C., Lu, F.J., Huang, C.F., Chen, W.K., Liu, S.H., Lin-Shiau, S.Y., 2007. The diabetogenic effects of the combination of humic acid and arsenic: in vitro and in vivo studies. Toxicol. Lett. 172, 91-105.

Yuan, H., Zhang, X., Huang, X., Lu, Y., Tang, W., Man, Y., Wang, S., Xi, J., Li, J., 2010. NADPH oxidase 2-derived reactive oxygen species mediate FFAs-induced dysfunction and apoptosis of beta-cells via JNK, p38 MAPK and p53 pathways. PLoS One 5, e15726-e15734.

Yusuf, M., Fariduddin, Q., Hayat, S., Ahmad, A., 2011. Nickel: an overview of uptake, essentiality and toxicity in plants. Bull. Environ. Contam. Toxicol. 86, 1-17.

Figure Legends

Figure 1. Effects of $NiCl_2$ on cell viability in pancreatic islet β-cell-derived RIN-m5F cells. Cells were cultured in 24-well plates and treated with $NiCl_2$ (0 - 325 μg/mL) for 24 h, and cell viability was determined by MTT assay. Data are presented as mean ± S.D. from four independent experiments with triplicate. *$p < 0.05$ as compared with vehicle control.

Figure 2. $NiCl_2$-triggered apoptotic effects in RIN-m5F cells. (A) Cells were treated with $NiCl_2$ (0, 130, and 195 μg/mL) for 24 h, and genomic DNA fragmentation (sub-G1 DNA content) were determined by flow cytometry. (B) RIN-m5F cells were treated with $NiCl_2$ (0, 130, and 195 μg/mL) for 24 h in the absence or present of JNK inhibitor (SP600125, 10 μM), and apoptotic cells were observed by fluorescence microscopy (Leica DMIL inverted microscope equipped with a charged-coupled device camera, 200×) staining with fluorescent probes: 6-CFDA (green fluorescence) and Ann Cy3 (red fluorescence) (a, control; b, $NiCl_2$-130 μg/mL; c, $NiCl_2$-195 μg/mL; d, SP600125-10 μM; e, $NiCl_2$-130 μg/mL + SP600125; f, $NiCl_2$-195 μg/mL + SP600125) as described in Materials and Methods secretion. Data in A are presented as mean ± S.D. from four independent experiments with triplicate. *$p < 0.05$ as compared with vehicle control.

Figure 3. Analysis of mitochondrial dysfunction in $NiCl_2$-treated RIN-m5F cells. Cells were treated with $NiCl_2$ (0, 130, and 195 μg/mL) for 24 h in the absence or presence of JNK inhibitor (SP600125, 10 μM). (A) Mitochondrial membrane potential depolarization was determined by flow cytometry analysis. (B) Cytosolic cytochrome

c release was analyzed by western blot. (C) The expression of pro-apoptotic genes (*bak* and *bid*) was examines by quantitative real-time PRC as described in the Materials and Methods secretion. Data in B, a representative result is shown (*a.*), and the intensity of bands is expressed as mean ± S.D. relative to the control of triplicate experiments after normalizing the bands to β-actin (*b.*). Results shown in A and C are presented as from four independent experiments with triplicate. $*p < 0.05$ as compared with vehicle control. $^{\#}p < 0.05$ as compared with $NiCl_2$-alone.

Figure 4. $NiCl_2$ induced caspase cascades activation and cleavages of poly (ADP-ribose) polymerase (PARP) in RIN-m5F cells. Cells were treated with $NiCl_2$ (0, 130, and 195 μg/mL) for 24 h in the absence or presence of JNK inhibitor (SP600125, 10 μM), and the expressions of active form caspase-9 (A), caspase-7 (B), caspase-3 (C), and PARP cleavage (D) were examine by western blot analysis as described in Materials and Methods secretion.

Figure 5. Effects of $NiCl_2$ on mitogen-activd protein kinase (MAPK) activation in RIN-m5F cells. (A) Cells were treated with $NiCl_2$ (0, 130, and 195 μg/mL) for 15 and 30 min, and detected the phosphorylation of c-jun N-terminal kinase (JNK)-1/2, the extracellular signal-regulated kinase (ERK)-1/2, and p38-MAPK were detected by western blotting analysis. (B) RIN-m5F cells were treated with $NiCl_2$ (0, 130, and 195 μg/mL) for 30 mins in the absence or presence of JNK inhibitor (SP600125, 10 μM), the phosphorylation of JNK-MAPK protein was examined.

Figure 6. Schematic diagram of the signal pathways involved in Ni-induced apoptosis in pancreatic islet β-cells. Proposed models represent that Ni causes the pancreatic β-cell apoptosis through JNK1/2-MAPK regulated mitochondria-dependent apoptotic

signaling cascades.

Figure
Click here to download Figure: Ni and RIN-m5F cell for Toxicology-total figures.pdf

Figure 1

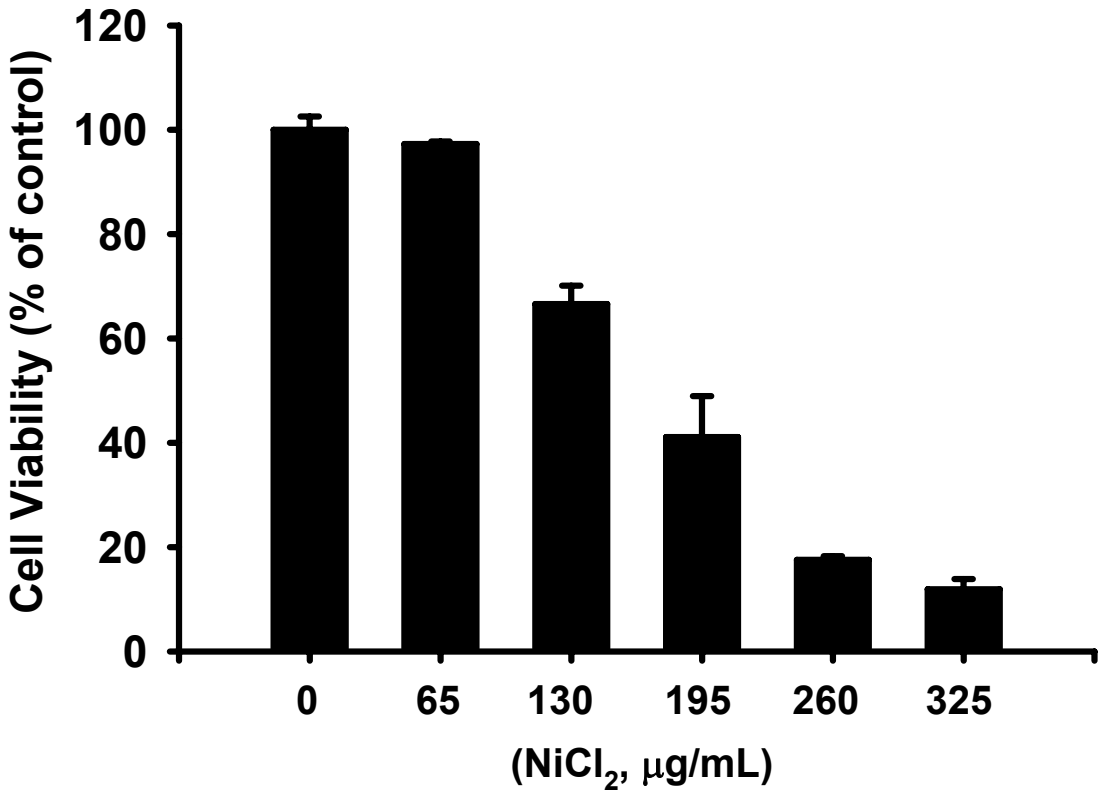


Figure 2

(A).

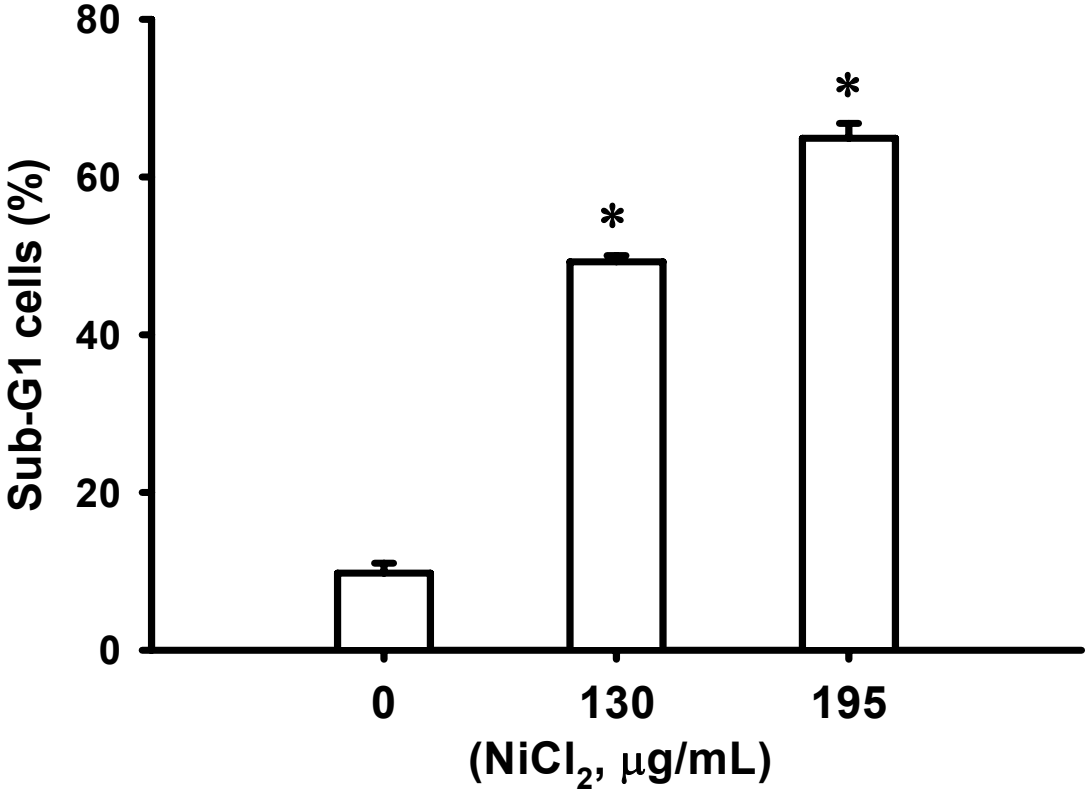


Figure 2

(B).

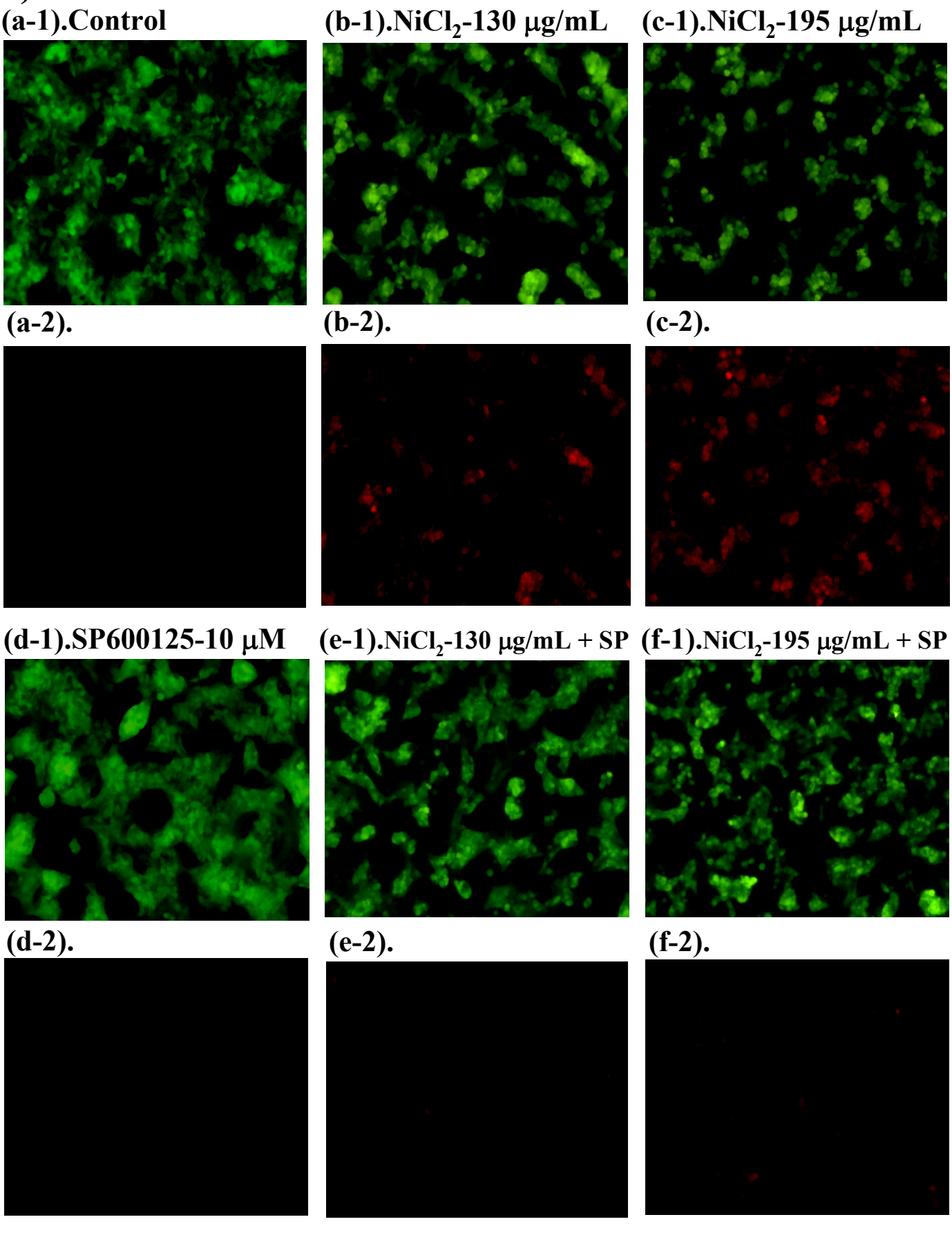

(a-1).Control
(b-1).NiCl2-130 μg/mL
(c-1).NiCl2-195 μg/mL
(a-2).
(b-2).
(c-2).
(d-1).SP600125-10 μM
(e-1).NiCl2-130 μg/mL + SP
(f-1).NiCl2-195 μg/mL + SP
(d-2).
(e-2).
(f-2).

Figure 3

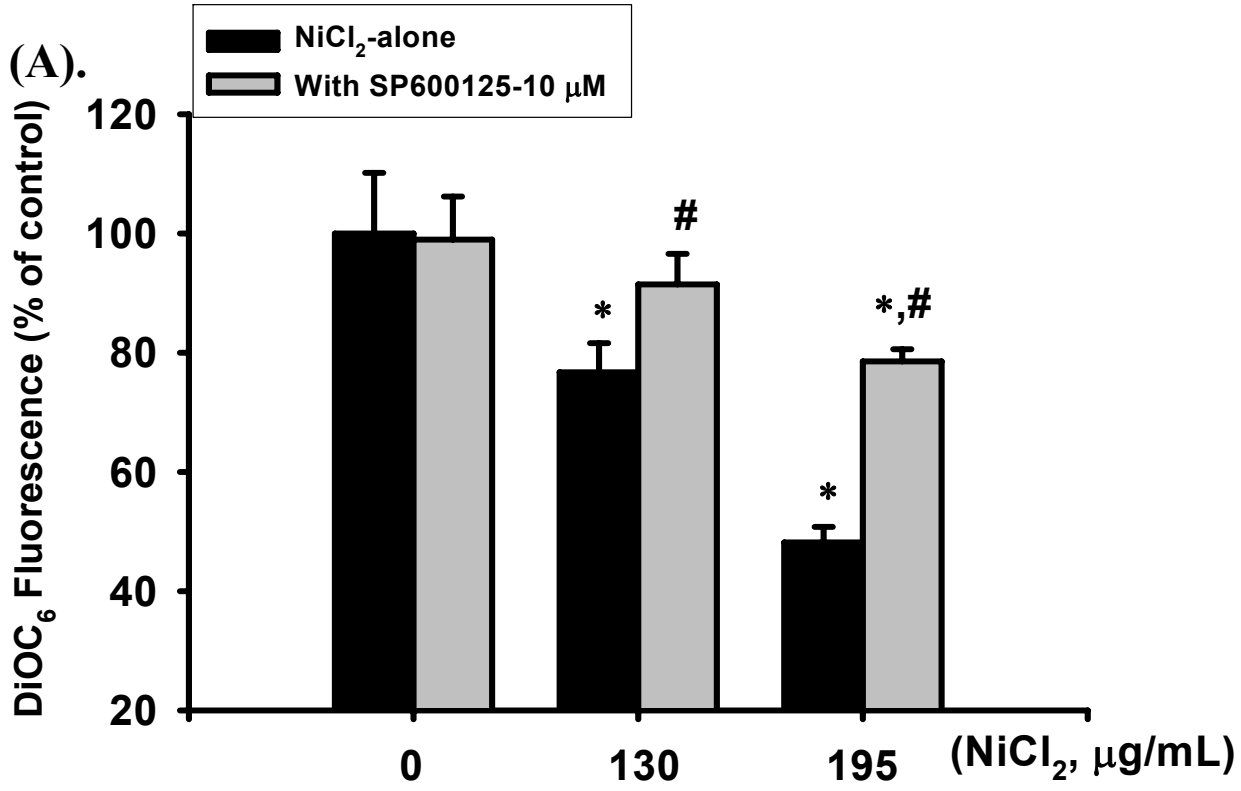
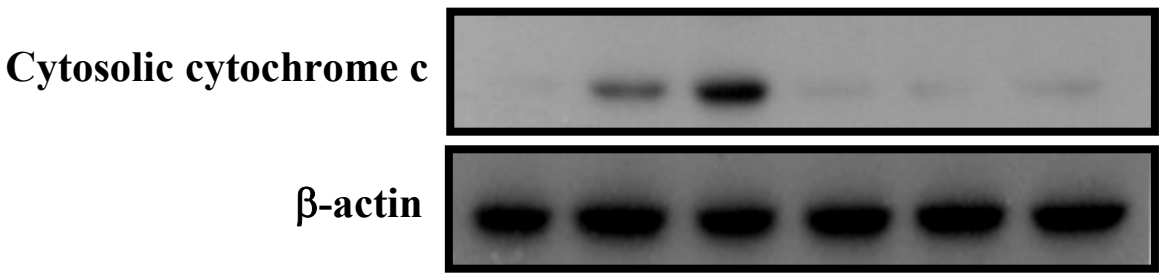

(A).
NiCl2-alone
With SP600125-10 μM
DiOC6 Fluorescence (% of control)
120
100
80
60
40
20
*
#
*,#
*
0
130
195
(NiCl2, μg/mL)

Figure 3

(B).

a.


NiCl2 (μg/mL) 0 130 195 0 130 195
SP600125 (μM) 0 0 0 10 10 10
Cytosolic cytochrome c
β-actin

b.

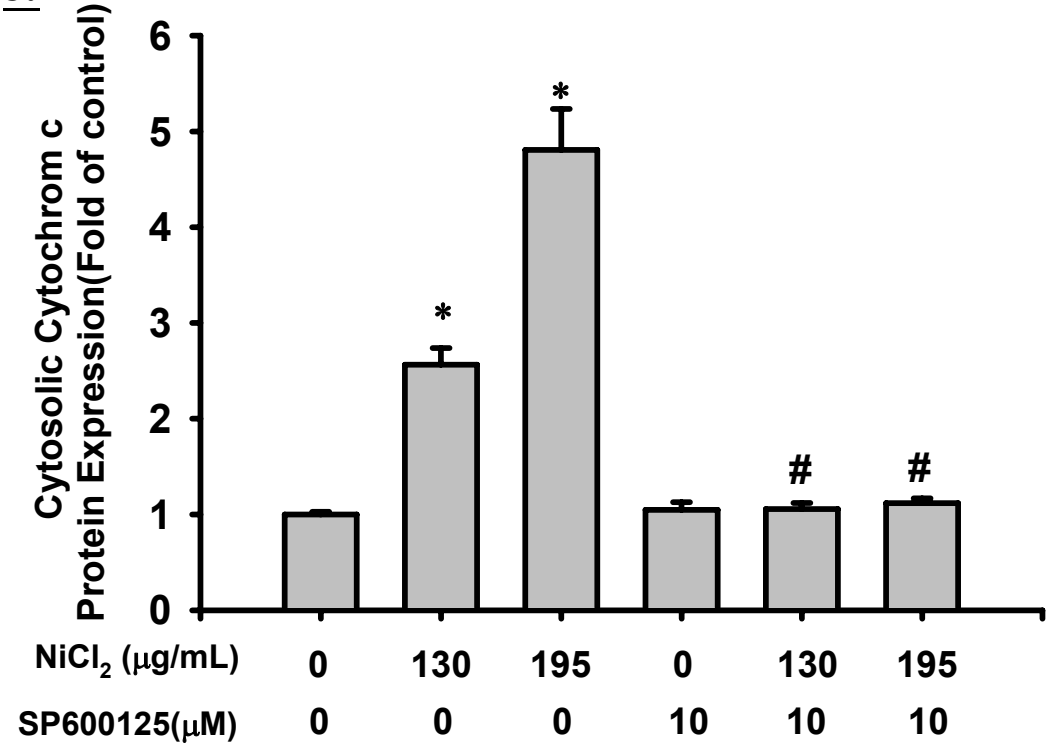

Cytosolic Cytochrom c
Protein Expression(Fold of control)
0
1
2
3
4
5
6
*
*
#
#
NiCl2 (μg/mL) 0 130 195 0 130 195
SP600125(μM) 0 0 0 10 10 10

Figure 3

(C).

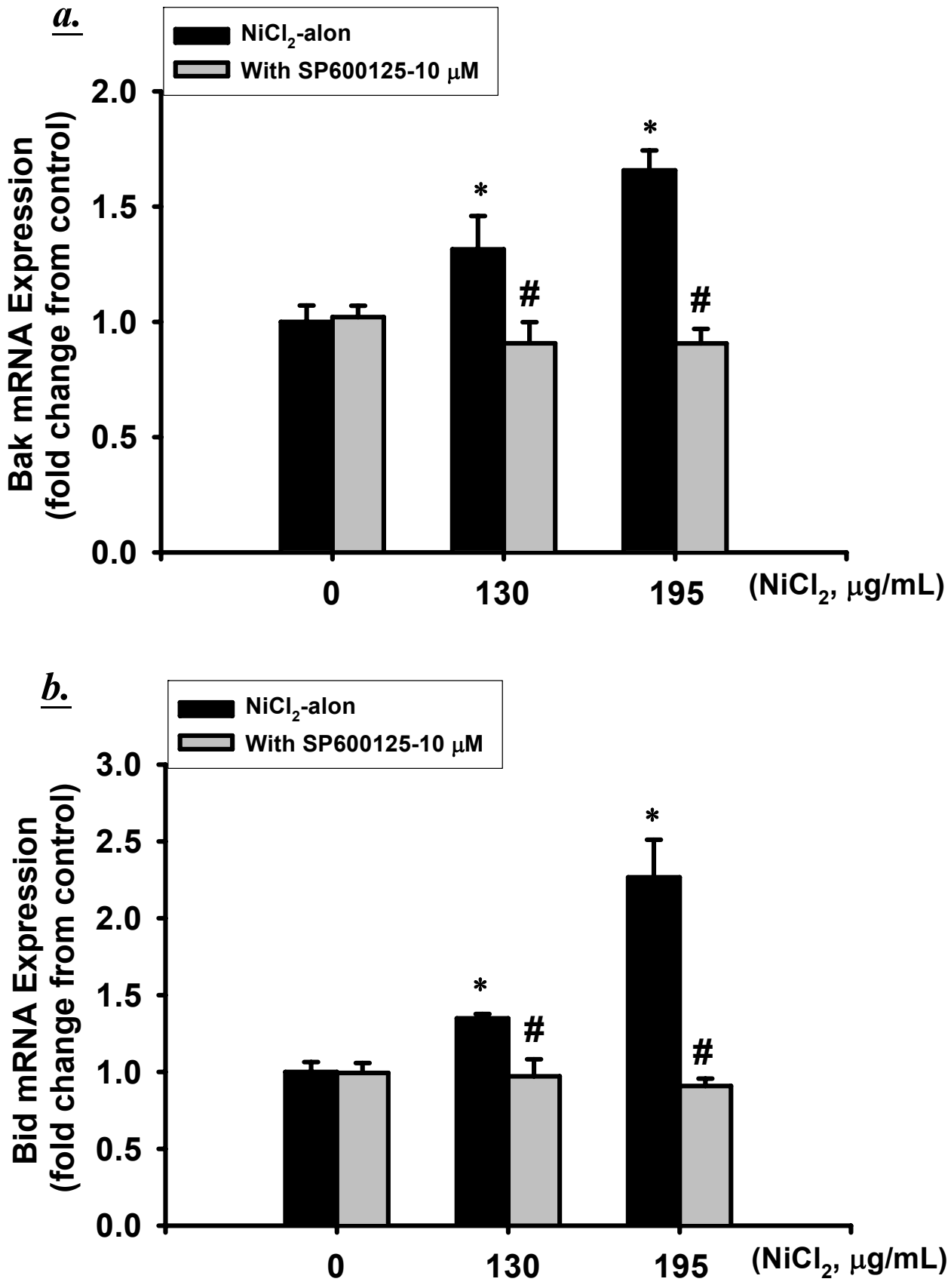

a.
NiCl2-alon
With SP600125-10 μM
Bak mRNA Expression
(fold change from control)
2.0
1.5
1.0
0.5
0.0
*
*
#
#
0
130
195
(NiCl2, μg/mL)
b.
NiCl2-alon
With SP600125-10 μM
Bid mRNA Expression
(fold change from control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
#
#
0
130
195
(NiCl2, μg/mL)

Figure 4

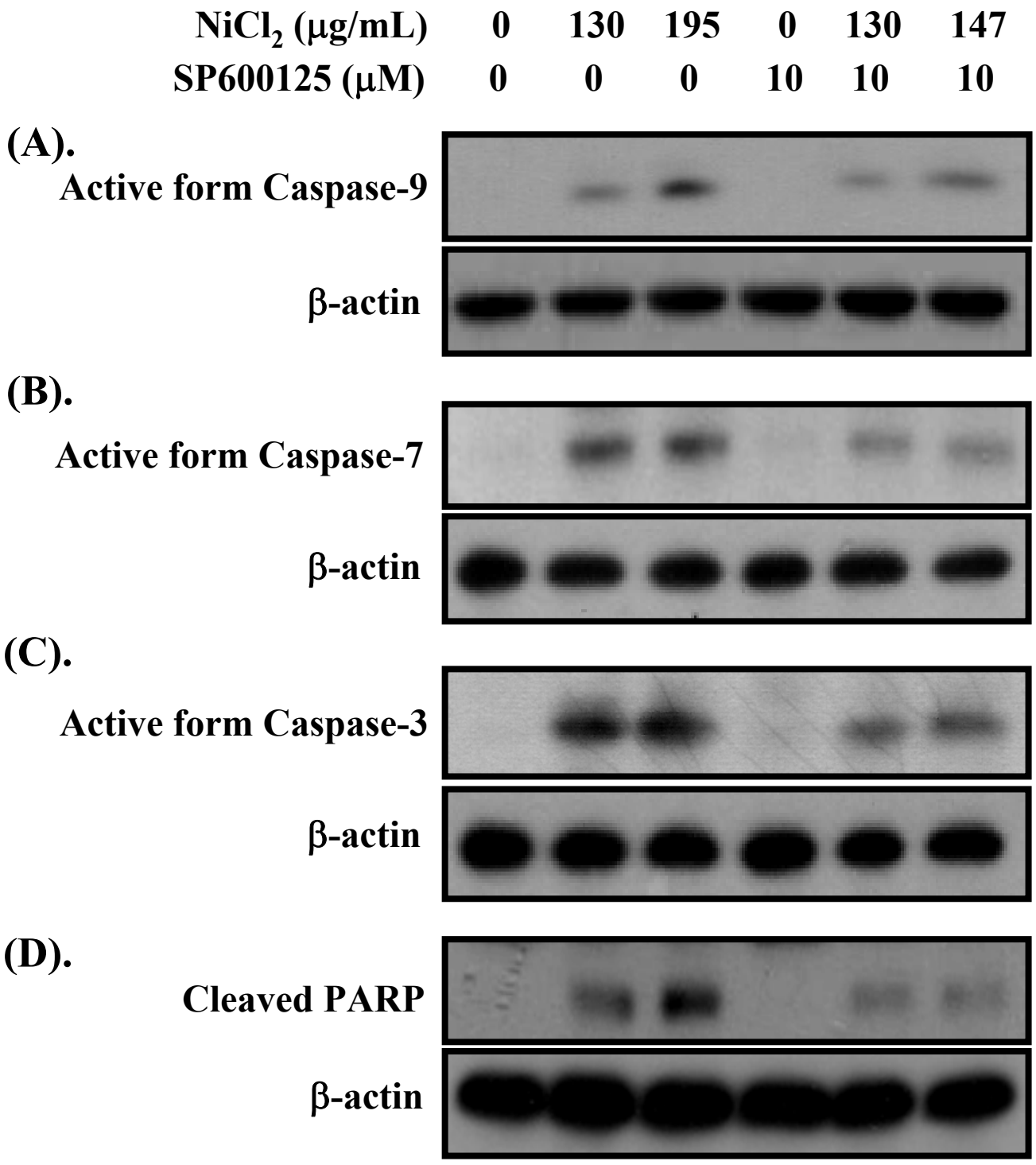

NiCl2 (μg/mL) 0 130 195 0 130 147
SP600125 (μM) 0 0 0 10 10 10
(A).
Active form Caspase-9
β-actin
(B).
Active form Caspase-7
β-actin
(C).
Active form Caspase-3
β-actin
(D).
Cleaved PARP
β-actin

Figure 5

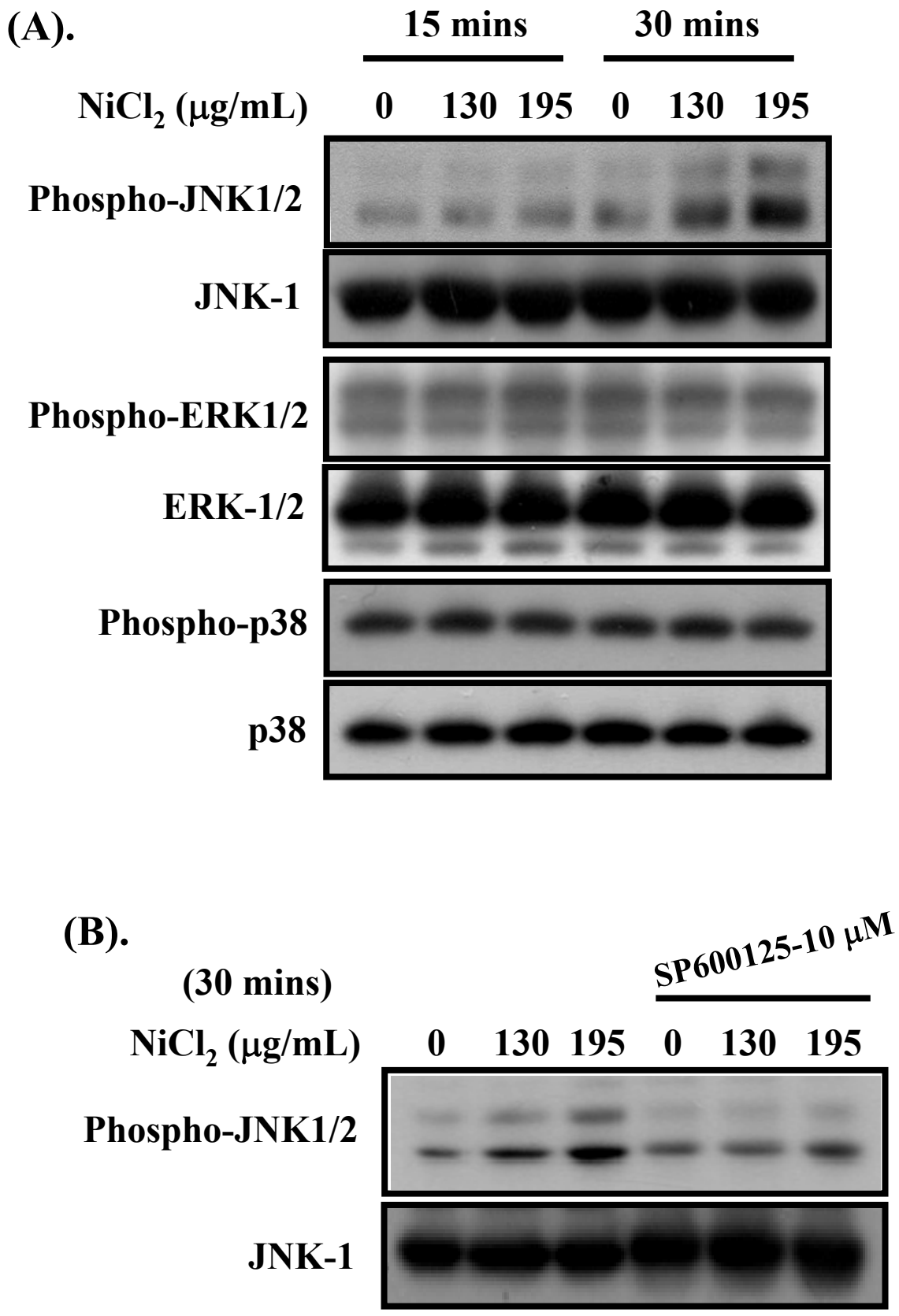
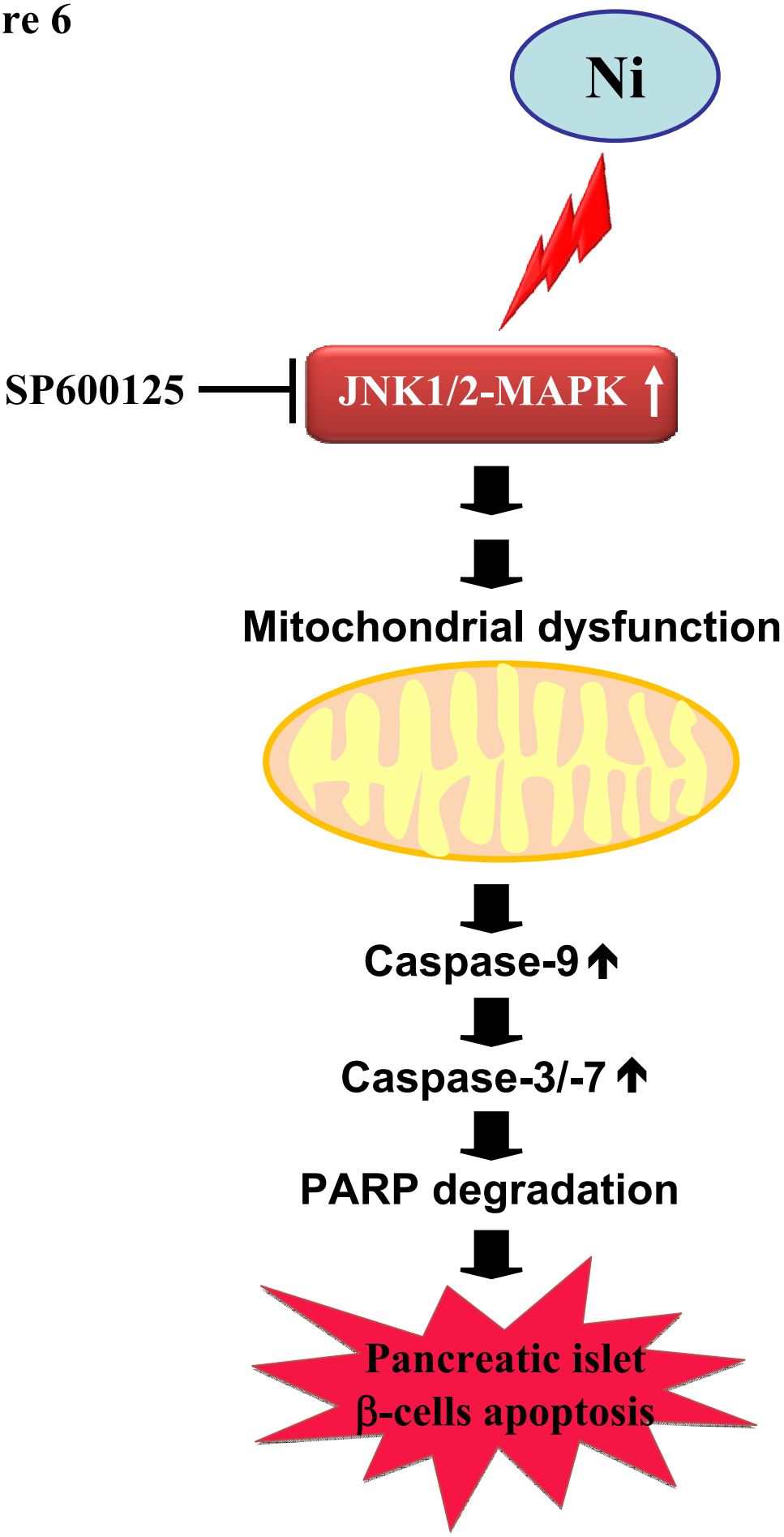

(A).
15 mins
30 mins
NiCl2 (μg/mL)
0 130 195 0 130 195
Phospho-JNK1/2
JNK-1
Phospho-ERK1/2
ERK-1/2
Phospho-p38
p38
(B).
(30 mins)
SP600125-10 μM
NiCl2 (μg/mL)
0 130 195 0 130 195
Phospho-JNK1/2
JNK-1

Figure 6


Ni
SP600125
JNK1/2-MAPK
Mitochondrial dysfunction
Caspase-9
Caspase-3/-7
PARP degradation
Pancreatic islet
β-cells apoptosis

*Conflict of Interest Statement

Toxicology

Conflict of Interest Policy

Manuscript number (if applicable):

Article Title: Nickel(II) induced JNK activation-regulated mitochondria-dependent apoptotic pathway leading to cultured rat pancreatic β-cell death

Author name: Hsi-Chin Wu, Ching-Yao Yang, Dong-Zong Hung, Chin-Chuan Su, Kuo-Liang Chen, Cheng-Chien Yen, Tsung-Jung Ho, Yi-Chang Su, Chun-Fa Huang, Chun-Hung Chen, Ling-Mei Tsai, Ya-Wen Chen

Declarations

Toxicology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

Please declare any financial or personal interests that might be potentially viewed to influence the work presented. Interests could include consultancies, honoraria, patent ownership or other. If there are none state 'there are none'.

Please state any competing interests

All authors declare that there are no conflicts of interest in this study.

Funding Source

All sources of funding should be acknowledged and you should declare any extra funding you have received for academic research of this work. If there are none state 'there are none'.

Please state any sources of funding for your research

This work was supported by research grants form the National Science Council of Taiwan (NSC 98-2314-B-039-015 and NSC 98-2320-B-039-014-MY3), the National Health Research Institutes of Taiwan (NHRI-EX98-974SI and NHRI-EX99-9744SI), and the China Medical University, Taichung, Taiwan (CMU-99-TC-27). It was also supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004).

Signature (a scanned signature is acceptable, but each author must sign)	**Print name**
His Chin Wu	His-Chin Wu
Ching-Yao Yang	Ching-Yao Yang
Chin-Chuan Su	Chin-Chuan Su
Dong-Zong Hung	Dong-Zong Hung
Kuo-Liang Chen	Kuo-Liang Chen
Cheng-Chien Yen	Cheng-Chien Yen
Tsung-Jung Ho	Tsung-Jung Ho
Yi-Chang Su	Yi-Chang Su
Chun-Fa Huang	Chun-Fa Huang
Chun-Hung Chen	Chun-Hung Chen
Ling-Mei Tsai	Ling-Mei Tsai
Ya-Wen Chen	Ya-Wen Chen