กรดแอสคอร์บิกลดความเป็นพิษต่อเซลล์และความบกพร่องในการหลั่งอินซูลินของเซลล์เบต้าตับอ่อนชนิด RIN-m5F จากการเหนี่ยวนำด้วยแคดเมียมไนเตรต

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บทคัดย่อ

การได้รับสารแคดเมียมมีความเกี่ยวข้องกับความผิดปกติของโรคเมตาบอลิซึม เช่น โรคเบาหวานชนิดที่ 2 โดยกระตุ้นให้เกิดภาวะเครียดออกซิเดชันและทำให้การทำงานของเซลล์เบต้าของตับอ่อนบกพร่อง งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษากลไกความเป็นพิษของแคดเมียมไนเตรตในเซลล์เบต้าตับอ่อนชนิด RIN-m5F และประเมินฤทธิ์ป้องกันของกรดแอสคอร์บิก ผลการศึกษาพบว่าแคดเมียมลดการรอดชีวิตของเซลล์แบบแปรผันตามความเข้มข้น โดยพบความเป็นพิษอย่างมีนัยสำคัญตั้งแต่ 5 μM ขึ้นไป และมีค่า IC50 เท่ากับ 1.7 μM ที่ระดับดังกล่าว แคดเมียมเพิ่มระดับสารอนุมูลอิสระภายในเซลล์ (ROS) และการแสดงออกของยีนที่เกี่ยวข้องกับภาวะเครียดออกซิเดชัน (Nox1 และ Nox4) และยีนส่งเสริมการตายของเซลล์ (Bax และ p53) ขณะที่ลดการแสดงออกของยีน Bcl-2 และ pre-insulin การให้กรดแอสคอร์บิกขนาด 100 μM ล่วงหน้าช่วยลดการสะสมของ ROS และลดการแสดงออกของยีนที่เกี่ยวข้องกับความเครียดออกซิเดชันและการเกิดอะพ็อพโทซิส ในด้านการทำงานแคดเมียมลดการหลั่งอินซูลินเมื่อถูกกระตุ้นด้วยกลูโคส (GSIS) อย่างเด่นชัด โดยระดับ GSIS ลดจาก 6.07 ± 0.91 ng/mL ในกลุ่มควบคุม เหลือ 1.43 ± 0.21 ng/mL และค่าดัชนีกระตุ้นลดลงเหลือ 1.74 ± 0.26 การให้กรดแอสคอร์บิกช่วยฟื้นฟูระดับ GSIS เป็น 3.59 ± 0.54 ng/mL และเพิ่มค่า SI เป็น 3.95 ± 0.59 ผลการศึกษานี้ชี้ให้เห็นว่ากรดแอสคอร์บิกช่วยปกป้องเซลล์เบต้าจากความเป็นพิษของแคดเมียม โดยลดภาวะเครียดออกซิเดชันและการตายของเซลล์ รวมทั้งช่วยคงสมรรถภาพการหลั่งอินซูลินของเซลล์เบต้าได้

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Ascorbic Acid Attenuates Cadmium Nitrate-Induced Cytotoxicity and Insulin Secretion Deficits in Pancreatic Beta Cells (RIN-m5F)

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Abstract

Cadmium exposure contributes to metabolic disorders, such as type 2 diabetes mellitus, by inducing oxidative stress and impairing pancreatic beta cell's function. This study investigated the cytotoxic mechanisms of cadmium nitrate in beta cells (RIN-m5F) and evaluated the protective efficacy of ascorbic acid. Cadmium nitrate treatment resulted in a dose-dependent reduction in cell viability (significant at ≥ 5 μM). At the half maximal inhibitory concentration (IC50) of 1.7 μM, cadmium significantly elevated intracellular reactive oxygen species (ROS) and upregulated oxidative stress markers (Nox1 and Nox4) and pro-apoptotic genes (Bax and p53), while downregulating Bcl-2 and pre-insulin expression. Pretreatment with 100 μM ascorbic acid effectively mitigated ROS accumulation and reversed these apoptotic and oxidative gene expression profiles. Functionally, cadmium exposure severely compromised glucose-stimulated insulin secretion (GSIS), reducing levels to 1.43 ± 0.21 ng/mL compared to 6.07 ± 0.91 ng/mL in controls, and suppressed the stimulation index to 1.74 ± 0.26. Ascorbic acid pretreatment significantly attenuated these deficits, partially restoring GSIS (3.59 ± 0.54 ng/mL) and improving the SI to 3.95 ± 0.59. These findings provide mechanistic evidence that ascorbic acid protects beta cells against cadmium-induced toxicity by suppressing oxidative stress and pro-apoptosis genes, thereby preserving insulin secretory function.

Keywords: Cadmium nitrate, Ascorbic acid, Oxidative stress, Apoptosis, Pancreatic beta cells, Insulin secretion

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Introduction

Cadmium (Cd) is a widespread environmental pollutant with well-documented toxicological effects in humans and animals. Chronic exposure to cadmium, primarily through contaminated food, water, and air, is associated with damage to multiple organ systems, including the kidneys, liver, lungs, and endocrine pancreas. Major sources of human exposure include cigarette smoke, industrial emissions (such as from nickel-cadmium battery manufacturing), and dietary intake via cereals, vegetables, and meat products. Notably, higher daily Cd intake has been reported in eastern Asian countries, including China, Japan, Vietnam, and Thailand. Due to its low clearance rate, Cd bio-accumulates in various organs, resulting in a biological half-life in humans of approximately 10–30 years[1]. While the hepatic and renal toxicity of Cd salts, such as cadmium nitrate ($Cd(NO_3)_2$), one of the major toxic products in the cigarette smoke, is well-established[2], their specific impact on the pancreas remains less understood.

Recent epidemiological studies have linked Cd exposure to impaired glucose homeostasis and an increased risk of developing type 2 diabetes mellitus (T2DM)[3, 4]. Chronic smokers, in particular, exhibit a higher risk of insulin resistance and T2DM[5]. Pancreatic beta cells, which are responsible for insulin synthesis and secretion, are particularly vulnerable to oxidative damage due to their inherently low antioxidant defenses. Cd compounds, such as $Cd(NO_3)_2$, have been shown to induce the overproduction of reactive oxygen species (ROS)[6], leading to oxidative stress, mitochondrial dysfunction, and apoptosis in various cell types[7-11]. However, the specific cytotoxic mechanism of $Cd(NO_3)_2$ on pancreatic beta cells remains insufficiently characterized[12].

Ascorbic acid acts as a potent antioxidant defense system, extending its utility beyond simple radical scavenging to the activation of endogenous protective networks. By enhancing antioxidant enzyme activity and protecting bio-molecules such as lipids, proteins, and DNA, ascorbic acid plays a vital role in mitigating oxidative injury[13]. Recent studies indicate that ascorbic acid treatment can reduce degenerative changes and improve pancreatic function in rat models of Cd toxicity[14]. However, the molecular mechanisms underlying the protective effects of ascorbic acid against Cd-induced beta cells damage have not been revealed.

In this study, we utilized the RIN-m5F cell line a widely accepted in vitro model for beta-cell function to investigate the cytotoxicity, oxidative stress and impairment of insulin secretion induced by

$Cd(NO_3)_2$. Furthermore, we aimed to elucidate the mechanistic protective effects of ascorbic acid in mitigating this toxicity.

Materials and Methods

Cell culture

RIN-m5F pancreatic beta cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 culture medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin to prevent bacterial contamination. The cells were incubated at 37°C in a humidified incubator with 5% carbon dioxide (CO_2) to maintain physiological pH and gas exchange. Sub-culturing was performed when cells reached approximately 70–80% confluency to ensure optimal growth conditions and to prevent contact inhibition[15]. Cell culture procedures followed the guidelines provided by the American Type Culture Collection (ATCC).

Cadmium nitrate treatment

Cadmium nitrate tetrahydrate [$Cd(NO_3)_2 \cdot 4H_2O$] was solubilized in dimethyl sulfoxide (DMSO) to prepare a concentrated stock solution 10 mM. This stock solution was subsequently diluted with complete culture medium to achieve the requisite working concentrations. All preparations were conducted under aseptic conditions, and solutions were utilized immediately for cellular treatment.

MTT assay for cell viability

Cell viability was evaluated using the MTT assay as described by Mosmann (1983)[16]. RIN-m5F cells were seeded in 96 well plates at a density of 1×10^5 cells per well and treated with $Cd(NO_3)_2$ or ascorbic acid at final working concentrations of 0.1, 1, 5, 10, 50, and 100 µM for 24 hours, respectively. After treatment, 20 µL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 4 hours at 37°C. Formazan crystals formed were dissolved by adding 100 µL of DMSO to each well, and absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated as a percentage relative to untreated control cells.

ROS generation assay

Intracellular ROS levels were measured using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) following the method of Hempel and Lademann (2002)[17]. RIN-m5F cells were seeded at a density of 5×10^5 per well in 96 well plates and treated with $Cd(NO_3)_2$ for 24 hours. To assess the

protective effect of ascorbic acid, cells were pretreated with 100 µM ascorbic acid for 1 hour prior to $Cd(NO_3)_2$ exposure. After treatment, cells were incubated with 10 µM DCFH-DA for 30 minutes at 37°C in the dark. Following incubation, the cells were washed twice with phosphate-buffered saline (PBS) and then maintained in phenol-red free RPMI without fetal bovine serum. The results were analyzed immediately by using fluorescence microplate reader (Bio-Tek Instruments) with excitation and emission wavelengths of 485 nm and 530 nm, respectively. Hydrogen peroxide (H_2O_2) (50 µM) was used as positive control. After measurement of DCF fluorescence, MTT assay was performed immediately according to the procedures described above. Data were normalized to untreated control cells.

DCFH-DA and 4',6-diamidino-2-phenylindole (DAPI) staining for ROS detection

For qualitative evaluation of ROS generation, RIN-m5F cells were seeded at a density of 1×10^6 cells per well in 6-well plates. Cells were pretreated with 100 µM ascorbic acid for one hour prior to exposure to 1.7 µM $Cd(NO_3)_2$ (IC50) for 24 hours. H_2O_2 (50 µM) was used as positive control. Subsequently, cells were incubated with 10 µM DCFH-DA for 30 minutes. Following incubation, cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 1 µg/mL DAPI for five minutes to visualize nuclear morphology. ROS production was detected by green fluorescence emitted from DCFH-DA, while nuclear staining was observed as blue fluorescence from DAPI using fluorescence microscopy.[18]

RNA isolation and real-time PCR analysis

RIN-m5F cells were cultured at a density of 1×10^6 cells per well in 6-well plates and exposed to $Cd(NO_3)_2$ according to previously established experimental conditions. Total RNA was subsequently isolated using the TRI extraction reagent (Favorgen Biotech Corp., Ping-Tung, Taiwan), with slight modifications to the manufacturer's protocol. The extracted RNA was then reverse transcribed into complementary DNA (cDNA) using oligo(dT) primers and a commercial reverse transcription kit (Bioneer, Daejeon, Korea)[19], following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) analysis was performed using the Bio-Rad detection system with SYBR Green dye in 96-well plates. Gene expression levels of Bcl-2, Bax, p53[20], PRE-INS, NADPH oxidase isoforms (Nox1 and Nox4), and the housekeeping gene GAPDH were measured using specific

primers, as detailed in Table 1. Relative mRNA expression levels were normalized to GAPDH, and fold changes were determined using the 2^−ΔΔCt method.

Table 1. Specific primer pairs used to amplify gene elaborated in oxidative stress and apoptosis pathway

Pair	Name	Sequence 5'-3'	T (ºC)	Fragment size (bp)
1	Bcl-2 F	GTGGATGACTGAGTACCT	56	118
	Bcl-2 R	CCAGGAGAAATCAAACAGAG		
2	Bax F	CTACAGGGTTTCATCCAG	53	133
	Bax R	CCAGTTCATCTCCAATTCG		
3	p53 F	ACAGCGTGGTGGTACCGTAT	60	83
	p53 R	GGAGCTGTTGCACATGTACT		
4	Pre-INS-F	AGGCTTTTGTCAAACAGCACCTT	60	212
	Pre-INS-R	ATCCACAATGCCACGCTTCTG		
5	Nox1-F	CTTCCTCACTGGCTGGGATA	58	219
	Nox1-R	CGACAGCATTTGCGCAGGCT		
6	Nox4-F	GTTAAACACCTCTGTCTGCTTG	64	454
	Nox4-R	CACCTGTCAGGCCCGGAACA		
7	GAPDH-F	CAGGGCTGCCTTCTCTTGTG	57	359
	GAPDH-R	TCTCGTGGTTCACACCCATC		

Glucose-Stimulated Insulin Secretion (GSIS) Assay

RIN-m5F pancreatic beta-cells were cultured in 24 well plates to 80–90% confluence, corresponding to approximately 2.7×10^5 cells per well. Cells were exposed to 1.7 µM $Cd(NO_3)_2$ for 24 hours, in the presence or absence of a 1 hour pre-treatment with 100 µM ascorbic acid. Following treatment, cells were washed with Krebs–Ringer bicarbonate buffer (KRBB; 115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4) supplemented with 0.1% BSA, and pre-incubated in low-glucose KRBB (2.8 mM) for 1 hour. Subsequently, cells were incubated in KRBB containing either low (2.8 mM) or high (16.7 mM) glucose for 1 hour at 37°C. Supernatants were collected and stored at −80°C. Insulin concentrations were quantified using a rat insulin ELISA kit

(ERINS, Invitrogen), and the stimulation index (SI) was calculated as the ratio of insulin secretion under high- versus low-glucose conditions.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD) from at least three independent experiments, each performed in triplicate. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test, performed with GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). A p value of < 0.05 was considered statistically significant.

Results

Effect of cadmium nitrate on cell viability assessed by MTT assay

Treatment with increasing concentrations of $Cd(NO_3)_2$ led to a dose-dependent decrease in cell viability, as shown in Figure 1. Cells exposed to the negative control (0.1% DMSO) maintained high viability (98.63% ± 2.75), indicating negligible cytotoxicity from the solvent. In contrast, the positive control (100% DMSO) significantly reduced viability to 27.99% ± 3.06, confirming the assay's responsiveness to a known cytotoxic agent. At the lowest $Cd(NO_3)_2$ concentration tested (0.1 µM), cell viability was moderately reduced to 85.16% ± 4.15. A further decrease in cell viability was observed at 1 µM (74.03% ± 2.70), while a sharp decline in viability occurred at 5 µM (42.56% ± 3.40). Interestingly, this reduction plateaued at higher concentrations, with viabilities of 45.24% ± 1.40 at 10 µM, 43.84% ± 2.19 at 50 µM, and 34.59% ± 2.07 at 100 µM. These results indicate that $Cd(NO_3)_2$ exerts significant cytotoxic effects on cells at concentrations ≥ 5 µM, with limited additional impact at higher doses. Moreover, treatment with ascorbic acid at final working concentrations of 0.1, 1, 5, 10, 50, and 100 µM for 24 hours showed no significant difference in cell viability compared with the negative control, indicating that ascorbic acid exhibited no cytotoxic effects on the cells at concentrations up to 100 µM (Figure 1).

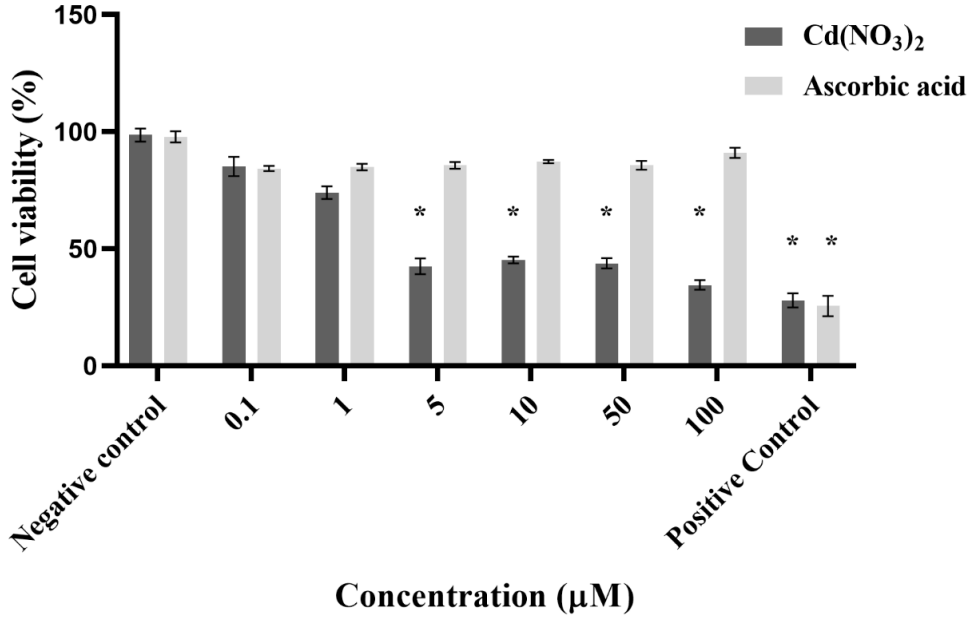

Figure 1. Effect of cadmium nitrate ($Cd(NO_3)_2$) on cell viability determined by the MTT assay. Cells treated with 0.1% DMSO served as the negative control, while 100% DMSO was used as the positive control. $Cd(NO_3)_2$ treatment induced dose-dependent cytotoxicity. In contrast, treatment with ascorbic acid for 24 h, even at concentrations up to 100 µM, did not result in a significant change in cell viability compared with the negative control. Statistical analysis; * Represents $p < 0.05$, compared to negative control group.

Effect of cadmium nitrate on intracellular ROS levels in RIN-m5F cells

The impact of $Cd(NO_3)_2$ on intracellular ROS generation and the potential protective effect of ascorbic acid were assessed in RIN-m5F cells. ROS levels were quantified using a DCF fluorescence assay and visualized through fluorescence microscopy (Figure 2). As shown in Figure 2 (left panel), exposure to cadmium nitrate 1.7 µM (IC50) for 24 hours significantly increased ROS production in RIN-m5F cells, as indicated by a marked elevation in DCF fluorescence compared to the negative control ($P < 0.001$). Pretreatment with 100 µM ascorbic acid for 1 hour prior to $Cd(NO_3)_2$ exposure significantly reduced DCF fluorescence relative to the $Cd(NO_3)_2$ only group ($P < 0.001$), indicating a protective antioxidant effect. H_2O_2 50 µM, used as a positive control, induced the highest level of ROS generation, consistent with its known oxidative properties. These findings were further supported by fluorescence microscopy (Figure 2, right panel). In control cells, minimal green fluorescence was observed, indicating low basal ROS levels. In contrast, $Cd(NO_3)_2$ treated cells

exhibited strong green fluorescence, reflecting elevated ROS production. Ascorbic acid pretreatment visibly reduced the intensity of green fluorescence, corroborating the quantitative assay results. Cells treated with H_2O_2 displayed intense green fluorescence throughout the field, confirming the assay's sensitivity to oxidative stress. Overall, these results demonstrate that $Cd(NO_3)_2$ induces significant oxidative stress in pancreatic beta cells, and that ascorbic acid effectively attenuates ROS accumulation under these conditions.

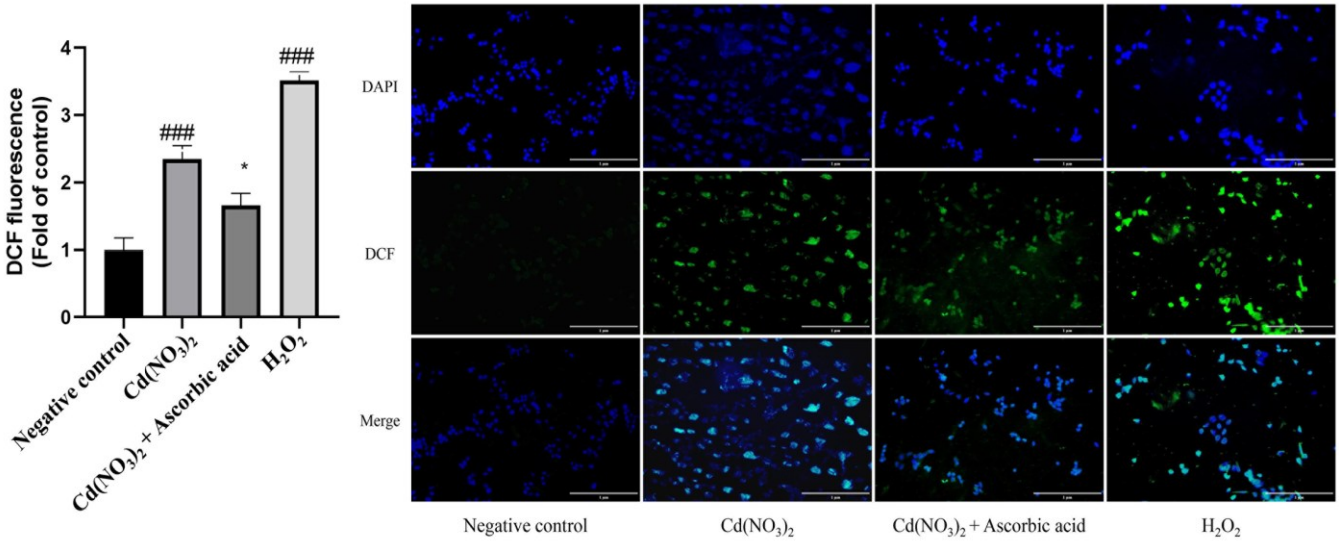

Figure 2. Effect of cadmium nitrate $Cd(NO_3)_2$ on intracellular reactive oxygen species (ROS) generation in RIN-m5F cells. (Left panel) Quantification of ROS levels was performed using a DCF fluorescence assay. Cells were treated with $Cd(NO_3)_2$, with or without 1 hour pretreatment with 100 µM ascorbic acid, followed by 24 hour exposure. Hydrogen peroxide (H_2O_2) 50 µM was used as a positive control. Data are expressed as DCF fluorescence (fold change relative to negative control) and represent mean ± SD from at least nine independent experiments. Statistical analysis: $^{\#}p < 0.001$ vs. negative control; $^{*}p < 0.001$ vs. $Cd(NO_3)_2$ treated group. (Right panel) Representative fluorescence microscopy images showing ROS generation, scale bar: 1 µm. DCF fluorescence (green) indicates ROS, and DAPI (blue) stains nuclei. Images confirm increased ROS in $Cd(NO_3)_2$ and H_2O_2 treated groups, while ascorbic acid pretreatment reduced ROS signal.

Effect of cadmium nitrate on gene expression in RIN-m5F cells

To investigate the molecular effects of $Cd(NO_3)_2$ exposure and the potential protective role of ascorbic acid, the mRNA expression levels of genes associated with apoptosis (Bcl-2, Bax, and p53), oxidative stress (Nox1 and Nox4), and insulin synthesis (pre-insulin) in RIN-m5F cells

was evaluated, as shown in Figure 3. Gene expression was quantified by real-time RT PCR and normalized to the control group. Exposure to $Cd(NO_3)_2$ for 24 hours significantly altered the expression of all analyzed genes. The anti-apoptotic gene Bcl-2 was markedly downregulated (0.46 ± 0.05 vs. 1.00 ± 0.10 in control, $p < 0.001$), whereas pro-apoptotic markers Bax and p53 were significantly upregulated (2.10 ± 0.21 and 1.85 ± 0.23, respectively; $p < 0.001$ and $p < 0.01$ compared to control). Genes involved in ROS production, Nox1 and Nox4, also showed substantial upregulation following cadmium exposure (2.43 ± 0.31 and 2.29 ± 0.25, respectively; $p < 0.001$). Additionally, pre-insulin mRNA expression was significantly decreased in the $Cd(NO_3)_2$-treated group (0.40 ± 0.05; $p < 0.001$). Pretreatment with 100 µM ascorbic acid for 1 hour prior to $Cd(NO_3)_2$ exposure attenuated these effects. Bcl-2 expression was partially restored (0.81 ± 0.05; $p < 0.01$ vs. Cd), while expression levels of Bax (1.40 ± 0.08), p53 (1.10 ± 0.10), Nox1 (1.30 ± 0.48), and Nox4 (1.60 ± 0.20) were significantly reduced compared to the $Cd(NO_3)_2$-only group ($p < 0.05$ to $p < 0.01$). Importantly, pre-insulin expression was also increased in the ascorbic acid pretreatment group (0.70 ± 0.05; $p < 0.001$ vs. Cd). These results indicate that $Cd(NO_3)_2$ induces oxidative stress and apoptosis gene expression while suppressing insulin gene expression in pancreatic beta cells. Ascorbic acid pretreatment exerts a protective effect by mitigating these molecular alterations.

Effect of cadmium nitrate on RIN-m5F cells insulin secretions

Under control conditions, RIN-m5F cells exhibited a robust response to glucose, yielding a stimulation index (SI) of 5.67 ± 0.85. Exposure to $Cd(NO_3)_2$ significantly impaired this function, drastically reducing basal insulin to 0.82 ± 0.12 ng/mL compared to 1.07 ± 0.16 ng/mL in control group and GSIS to 1.43 ± 0.21 ng/mL compared to 6.07 ± 0.91 ng/mL in controls ($p < 0.001$). Consequently, the SI in $Cd(NO_3)_2$ treated cells dropped markedly to 1.74 ± 0.26. However, pretreatment with ascorbic acid significantly attenuated this dysfunction, partially restoring GSIS (3.59 ± 0.54 ng/mL) and improving the SI to 3.95 ± 0.59 ($p < 0.01$ vs. $Cd(NO_3)_2$ alone), as shown in Table 2.

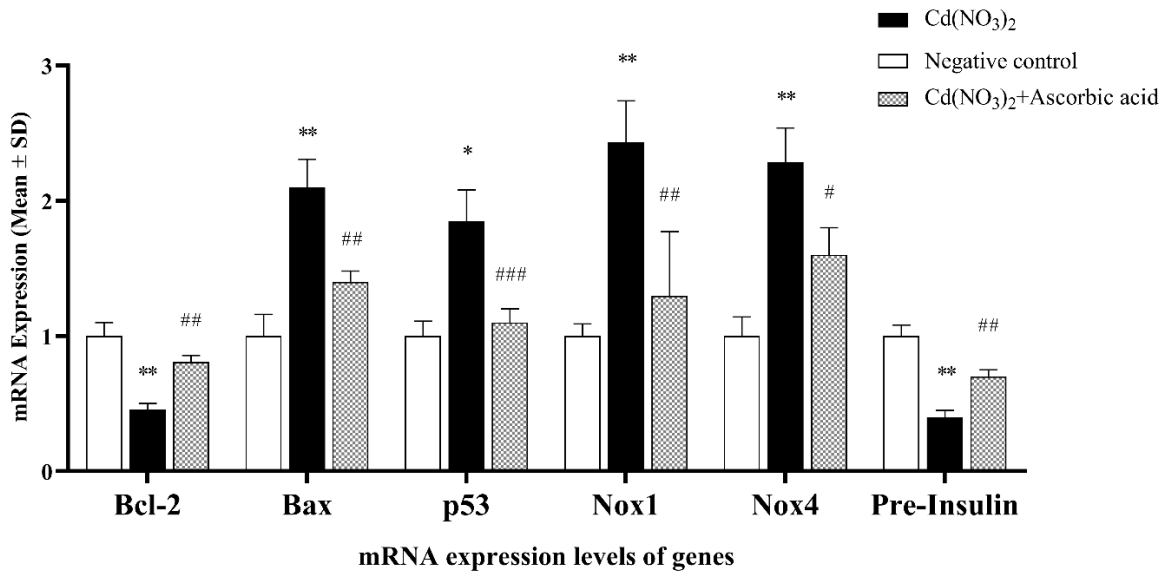

Figure 3. The mRNA expression levels of genes under different experimental conditions**.** Exposure to 1.7 µM cadmium nitrate ($Cd(NO_3)_2$) for 24 hours, significantly altered mRNA expression by downregulating Bcl-2 and pre-insulin and upregulating Bax, p53, Nox1, and Nox4 in RIN-m5F cells. Pretreatment with 100 µM ascorbic acid 1 hour prior to exposure to $Cd(NO_3)_2$ partially reversed these changes, indicating its protective effect against $Cd(NO_3)_2$ -induced oxidative stress and apoptosis. Statistical analysis: * $p < 0.01$, ** $p < 0.001$ vs. negative control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. $Cd(NO_3)_2$ -treated group.

Table 2. The effect of cadmium nitrate on the insulin secretion of RIN-m5F cells.

Group	Basal Insulin (ng/mL)	GSIS (ng/mL)	Stimulation Index (SI)
Control	1.07 ± 0.16	6.07 ± 0.91	5.67 ± 0.85
Cadmium nitrate 1.7 µM (24 hour)	0.82 ± 0.12 ***	1.43 ± 0.21 ***	1.74 ± 0.26 ***
Cadmium nitrate 1.7 µM + Ascorbic acid 100 µM (Pretreatment 1 hour)	0.91 ± 0.14	3.59 ± 0.54 ##	3.95 ± 0.59 ##

Data are mean ± SD (n = 3); *** $p < 0.001$ vs. control group, ## $p < 0.01$ vs. cadmium nitrate-treated group.

Discussion

The present study provides a mechanistic evaluation of the cytotoxicity of $Cd(NO_3)_2$ in RIN-m5F pancreatic beta cells, bridging the gap between epidemiological evidence linking environmental cadmium exposure to T2DM and cellular pathophysiology. A recent epidemiological

study in the Thai population revealed significant disparities in blood Cd concentrations between non-polluted and polluted areas. In men, mean levels ranged from 0.9 μg/L (non-polluted) to 6.9 μg/L (polluted), while in women, levels ranged from 0.8 μg/L to 5.2 μg/L[21]. These values approach or exceed the reference ranges established by the Centers for Disease Control and Prevention (CDC), which define typical unexposed levels as 0.1–4.0 μg/L and consider levels > 5.0 μg/L as a critical threshold for toxicity[22]. These benchmarks emphasize the clinical relevance of investigating cellular toxicity, as environmental exposures can elevate Cd levels toward harmful ranges. While previous studies have largely focused on cadmium chloride ($CdCl_2$), our investigation into $Cd(NO_3)_2$ a major constituent of cigarette smoke and agricultural runoff reveals a potent toxicity profile characterized by Nox-driven oxidative stress, mitochondrial apoptosis, and the transcriptional suppression of insulin.

The cell viability data (MTT assay) as indicated in Figure 1, demonstrated a sharp cytotoxic threshold at concentrations 5 μM. The observed half-maximal inhibitory concentration (IC50) was 1.7 μM, which is comparable to recent findings reported in the MIN6 pancreatic beta-cell line[23]. This high mortality rate aligns with the hypothesis presented in the introduction regarding the unique profile of $Cd(NO_3)_2$. Unlike the chloride salt, the nitrate moiety (NO_3^-) possesses intrinsic biological activity and can be metabolized into reactive nitrogen species (RNS). It is plausible that the severe toxicity observed in this study results from a synergistic effect as the accumulation of the cadmium ion (Cd^{2+}) depleting sulfhydryl pools, combined with the potential nitrosative stress from the nitrate group[24, 25]. This mirrors the complex chemical insults found in cigarette smoke and industrial effluents, suggesting that $Cd(NO_3)_2$ may be a more clinically relevant model for assessing pancreatic risk in smokers than $CdCl_2$.

This study demonstrated that pancreatic beta-cells, known for their weak intrinsic antioxidant defenses[26], respond to $Cd(NO_3)_2$ with a massive surge in ROS production (Figure 2). Markedly, genes expression analysis (Figure 3) identified the likely source of this stress. The significant upregulation of Nox1 and Nox4 indicates that Cd does not merely passively deplete antioxidants but actively stimulates ROS generation via the NADPH oxidase complex. This establishes a clear mechanistic link to the oxidative mechanisms, previously described as insufficiently characterized[27]. The ability of

ascorbic acid to blunt this ROS signal and downregulate Nox expression suggests that this oxidative loop is modifiable and not an irreversible consequence of metal accumulation. The transition from oxidative stress to cell death was confirmed to follow a mitochondria-dependent apoptotic pathway. The observed upregulation of p53 and Bax, concurrent with the downregulation of Bcl-2, creates a pro-apoptotic environment that favors mitochondrial outer membrane permeabilization, leading to the loss of cell viability. The p53 acts as a redox-sensitive transcription factor; its activation by Cd - induced ROS triggers the transcription of Bax, which subsequently perforates the mitochondria[28, 29]. This pathway highlights why mitochondrial dysfunction is a hallmark of Cd toxicity, as noted in previous broad-spectrum studies[14], and confirms its specific relevance to the pancreatic beta cells.

The specific mechanism by which ascorbic acid restores GSIS likely involves the preservation of mitochondrial bioenergetics. Insulin exocytosis is an energy-dependent process that requires a high ATP/ADP ratio to close K(ATP) channels and trigger membrane depolarization[30]. Cd-induced oxidative stress is known to disrupt the mitochondrial electron transport chain and dissipate the mitochondrial membrane potential, thereby severing the link between glucose sensing and insulin release[31]. By scavenging intracellular ROS, ascorbic acid protects mitochondrial integrity as evidenced by the reduced Bax/Bcl-2 ratio in our study (Figure 3) ensuring sufficient ATP generation to fuel the secretory machinery.

Furthermore, ascorbic acid may directly rescue the transcriptional machinery governing insulin synthesis. Our results showed that $Cd(NO_3)_2$ suppressed pre-insulin mRNA, a defect significantly reversed by ascorbic acid (Figure 3). It is well-established that key beta cells transcription factors, such as PDX-1 and MafA, are highly sensitive to oxidative stress; elevated ROS levels impair their DNA-binding activity and promote their degradation[32, 33] [3, 4]. Therefore, the antioxidant action of ascorbic acid likely stabilizes these factors, restoring pre-insulin gene expression and replenishing the insulin pool available for secretion.

Possibly the most clinically relevant finding is the functional paralysis of the surviving beta-cells. The significant collapse in the Stimulation Index (SI) (Table 2) and a suppression of pre-insulin mRNA. This dual defect reduced synthesis and reduced secretion provides a cellular basis for the insulin resistance and T2DM risk observed in chronic smokers and

nitrate-exposed populations[34, 35]. The loss of GSIS is likely secondary to the mitochondrial damage discussed above; without healthy mitochondria to generate ATP in response to glucose, the insulin secretion machinery fails[36, 37].

Finally, the comprehensive protection provided by ascorbic acid validates the oxidative etiology of this toxicity. Ascorbic acid did not merely improve survival; it restored the functional identity of the cells (pre-insulin expression and GSIS) and renormalized the apoptotic gene profile (Bax/Bcl-2 ratio). These findings offer a molecular rationale for the beneficial effects of ascorbic acid against $Cd(NO_3)_2$ induced cellular damage.

Conclusion

The present study demonstrates that $Cd(NO_3)_2$ exerts significant cytotoxic effects on RIN-m5F pancreatic beta cells in a dose-dependent manner, primarily through the induction of oxidative stress and upregulated pro-apoptosis gene expression. $Cd(NO_3)_2$ exposure markedly increased intracellular ROS levels, altered the expression of key pro- and anti-apoptotic genes (e.g., Bax, Bcl-2, and p53), upregulated oxidative stress markers (Nox1 and Nox4), and suppressed pre-insulin gene expression and reduced insulin secretion, suggesting a detrimental impact on both cell viability and beta-cell function. Importantly, pretreatment with ascorbic acid significantly attenuated $Cd(NO_3)_2$ - induced ROS accumulation, reduced the expression of pro-apoptotic and oxidative stress–related genes, restored Bcl-2 and pre-insulin expression levels, and improved insulin secretion and cell viability. Overall, this study provides mechanistic insights into the protective effects of ascorbic acid against $Cd(NO_3)_2$ induced cellular damage.

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Conflict of interest

The authors declare that there is no conflict of interest.

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