Vitamin C Protects against INS832/13 b-Cell Death and/or Dysfunction Caused by Glucolipotoxicity or 3T3-L1 Adipocyte Coculture

Ruojuan Wang1, Jia Liu2, Xiaoxuan Guo1, Fengyi Gao1, Baoping Ji1,* and Feng Zhou1

1 Beijing Key Laboratory of Functional Food from Plant Resources, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, 100083, P.R. China
2 China National Research Institute of Food and Fermentation Industries, Beijing, P.R. China

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Summary In diabetic patients, glucolipotoxicity induces multiple defects in b-cells. Furthermore, increasing evidence also confirms the direct interaction between the adipocytes and b-cells. The beneficial efficacy of vitamin C (Vc) on b-cells is rarely investigated. In this study, INS-1 832/13 b-cells were cultured with high levels of glucose and free fatty acid (FFA) or cocultured with 3T3-L1 adipocytes in the presence or absence of Vc. Vc decreased glucolipotoxicity-induced cell mass loss by reducing apoptosis and reactive oxidative species (ROS). After treatment with elevated glucose and FFA, b-cell secretion dysfunction was evidenced and was partially improved by 1, 10 and 50 μg/mL Vc treatment. In the coculture system, impaired secretion function was also moderately normalized upon addition of 10 and 50 μg/mL Vc to the coculture medium (p<0.05). Vc at 50 μg/mL significantly (p<0.05) inhibited the fatty acid release from adipocytes to the coculture medium. Meanwhile, the elevated ROS of cocultured b-cells was decreased in the presence of Vc (1 to 50 μg/mL). In both induction methods, intracellular TGs in both b-cells and adipocytes were decreased by Vc treatment; however, Vc did not affect the intracellular insulin level. Moreover, IL-6 and adiponectin levels in the coculture medium remained under the levels of the control group. The positive effects of Vc might be due to the antioxidant capacity and TG inhibitory effect of Vc.

Key Words vitamin C, b-cells, glucolipotoxicity, adipocyte, coculture

Several studies have shown that hyperglycemia is often accompanied by hyperlipidemia in type 2 diabetes mellitus (T2DM), and both play important roles in the development of T2DM (1). Chronic hyperglycemia and hyperlipidemia have been postulated to cause harm in the b-cells, commonly referred to as glucotoxicity and lipotoxicity (2, 3), respectively. It is suggested that glucotoxicity is partially mediated by the generation of chronic oxidative stress in b-cells (4) and lipotoxicity is probably caused by accumulation of a cytosolic signal derived from the fatty acid esterification pathway (5). The combination of glucotoxicity and lipotoxicity, referred to as glucolipotoxicity (5), leads to elevated insulin secretion at low glucose concentration, failure in responding to high glucose concentration, and a reduced insulin secretion index (ISI) (6, 7). Moreover, glucolipotoxicity causes less proinsulin biosynthesis, exhaustion of insulin stores, accumulation of TG deposits, and increased cell death by apoptosis (8).

Abundant evidence shows that in T2DM, adipocyte tissue not only liberates excessive free fatty acids (FFA), but also plays an important role in endocrine function,. It is reported that adipocyte can secrete leptin, resistin, adiponectin, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (9), which may affect the functions of pancreatic b-cells directly or indirectly. For instance, adiponectin protects MIN6 b-cells and INS-1 832/13 against apoptosis, and elevates the insulin gene expression and insulin secretion by mediating extracellular signal-regulated kinase and protein kinase B phosphorylation (10). Most of the previous studies focused on a single secretory factor; however, different secretory factors always co-exist and interact at the cellular level on the function of b-cells inside our bodies. Besides, there is a great deal of evidence indicating that adipocytes could surround and infiltrate the pancreas when diabetes occurs both in mice and humans (11, 12); therefore, the concentration of lipid metabolites and secretory products such as leptin and cytokines is likely to be high in the proximity of the islets.

Recently, the direct actions of adipocytes on b-cells has been unraveled by investigating cocultured adipocytes and pancreatic cells. After culturing with 3T3-L1 adipocytes, the stimulation index and mRNA levels of glucokinase, glucose transporter 2 and Kir6.2 genes in rat derived islets declined (13). After 1-wk coculturing, 3T3-L1 adipocytes were found to be capable of directly impairing insulin secretion, reducing intracellular calcium concentration and inhibiting expression of insulin
and glucokinase genes in MIN6 cells (14). Since the fat infiltration of the pancreas can be extensive in humans (12) and the close association of adipocytes might impair pancreatic cells directly, the relationship of islets and their nearby adipocytes which reflect the actual situation in vivo has attracted much attention.

It has been suggested that enhanced production of free radicals and oxidative stress from glucolipotoxicity is the central event in the development of diabetic complications. Boosting internal antioxidant defense systems by supplementing external antioxidants in cleaving free radicals may be helpful under certain circumstances. This might be especially vital for the pancreas, since it has a particularly weak intrinsic defense system against oxidative stress (15). Antioxidants, including vitamin C (Vc, ascorbic acid) and vitamin E, are well-known dietary antioxidants. Vc is a water-soluble essential vitamin with powerful antioxidant activity (16). A recent study on adults of European origin reported a significant inverse association between diabetes mellitus and plasma Vc levels (17) which suggests that Vc might be used to prevent the progression of diabetes and the occurrence of complications resulting from diabetes. Functions of Vc improving insulin action, glycemic control and endothelial function after treatment with Vc have also been documented (18, 19).

Although the protective effects of Vc are well investigated in animal and clinical trials involving pancreatitis and insulin resistance of T2DM, its direct efficacy on regulating β-cell functions is rarely studied. Moreover, to the best of our knowledge, there is no report focusing on the direct efficacy of Vc on impaired β-cells induced by glucolipotoxicity or adipocyte coculture. Therefore, the first goal of this work was to investigate the positive effect of Vc on the dysfunction and death of INS832/13 β-cells caused by high levels of glucose and FFA. Secondly, we examined the functional impact of Vc in an in vitro coculture system wherein INS832/13 β-cells were exposed to neighboring 3T3-L1 adipocytes.

MATERIALS AND METHODS

Chemicals. Oleic acid (OA), palmitic acid (PA), metformin, propidium iodide (PI), RNase, RPMI-1640, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), isobutylmethylxanthine (IBMX), dexamethasone (DEX), and bovine insulin were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and bovine serum albumin (BSA) were obtained from Gibco Life Technologies (Grand Island, NY). FFA-free bovine serum albumin (BSA) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Amino acid 3-[3-(hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Vc (free ascorbic acid) were purchased from Must Company (Chengdu, China). Bicinchoninic acid assay (BCA) kits were purchased from Beyotime (Haimen, China). An insulin radioimmunoassay (RIA) kit was obtained from Beijing Atom Hightech Company (Beijing, China). The TG analysis kits were supplied by JianCheng Company (Nanjing, China). The coculture system was from Corning (Corning, NY). Other reagents used were all of analytical grade.

BSA-bound FFA preparation. Stock solutions of FFA bound to BSA were prepared as previously described (20). The final molar ratio of FFA and BSA in stock solution was 6.6:1. Briefly, the OA and palmitate PA (molar ratio of 2:1) were completely dissolved in ethanol (final ethanol concentration in treatment solution was less than 0.1%). Then FFA-free BSA in RPMI-1640 medium was adjusted to a pH of 10 and applied to the FFA solution. After sonication for 40 min, the BSA-FFA medium pH was set to 7.4 and filtered through a 0.22 μm filter before stored at −20°C.

Cell culture. INS-1 832/13 cells (a kind gift from Christopher B. Newgard, Duke University, NC) were cultured in RPMI 1640 medium containing 11.1 mM glucose supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO2. The cells were seeded at a density of 2×10⁶ cells/mL and incubated at 37°C for 24 h. The cells were then washed with phosphate-buffered saline (PBS) and incubated with FBS-free RPMI-1640 with 5.6 mM glucose at 37°C for 12 h. After removal of the pretreated medium, the cells were incubated with 0.5% BSA in 5.6 mM glucose (Control), 22.4 mM glucose plus 0.5 mM FFA (Model), or 22.4 mM glucose plus 0.5 mM FFA with various concentrations of Vc for 36 h.

The 3T3-L1 preadipocyte cells were obtained from the ATCC (American Type Culture Collection, Manassas, VA) and maintained in DMEM supplemented with 10% BS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO2.

Cell viability assay. Cell viability was determined by MTT assay. Vc was dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 100 mM and was diluted to various concentrations with relative medium. The final concentration of DMSO was less than 0.1%. At the end of incubation in treatment solution (36 h or 48 h), the medium was replaced with 0.5 mg/mL MTT for 3 h in a humidified atmosphere (5% CO2, 37°C) in the dark. The MTT was removed and 150 μL DMSO was directly added into the wells. The absorbance of the reduced intracellular formazan product was read at 570 nm on a microplate plate reader (Spectra Max M2e, Molecular Devices, Sunnyvale, CA). The absorbance of the control group was regarded as 100% viability.

3T3-L1 preadipocyte differentiation assay and cocultured with β-cells. Cells were seeded at 5×10⁴ cells/mL in 6-well plates and were allowed to adhere for 2–3 d. On day 0, the medium was replaced with initiation media (DMEM with 10% BS, 0.5 mM IBMX, and 1 μM DEX). On day 2, the medium was replaced with progression media (DMEM with 10% BS and 10 μg/mL insulin). On day 4, the medium was replaced by DMEM with 10% FBS (adipocyte medium) and thereafter changed every 2 d with fresh medium until day 8. The Transwell system was applied in the coculture assay. After a 3T3-L1 differentiation period, the insert wells grown with INS-1 832/13
cells were inserted into the plate. The coculture medium was mixed 1:1 with complete culture medium of INS-1 832/13 cells and adipocytes. In the meantime, Vc was added at various concentrations and incubated for 48 h. The control groups were treated under the same conditions as the undifferentiated 3T3-L1 cells and without Vc treatment.

**Evaluation of insulin secretory function.** Insulin secretion in response to 2.8 or 16.7 mM glucose after 36 h Vc treatment was measured by an RIA kit. Cells were washed twice with HEPES balanced salt solution (HBSS, pH 7.4), and pre-incubated in HBSS buffer containing 2.8 mM glucose and 0.1% BSA for 2 h. Then the medium was replaced with HBSS buffer containing 2.8 mM or 16.7 mM glucose with 0.1% BSA and the cells were incubated sequentially for 2 h. The treatment medium was collected and kept at −80°C until tested. Results are expressed as insulin release in the medium (µIU/mg Pro·h). Basal insulin secretion (BIS) and glucose-stimulated insulin secretion (GSIS) are insulin release at 2.8 mM and 16.7 mM glucose, respectively. ISI, the ratio of GSIS to BIS, indicates the secretory function of pancreatic β-cells (7).

**Measurement of intracellular insulin contents.** Cells were cultured in 48-well plates as described above for 24 h. To extract insulin, cells were washed twice with PBS, followed by incubation in 400 µL acid alcohol solution (75 mL ethanol, 1.5 mL 12 M HCl, and 23.5 mL distilled water) overnight at 4°C. The solution was collected by centrifuging at 10,000 rpm for 5 min at 4°C, and the supernatants were stored at −80°C until insulin RIA assay. Cells cultured side by side under the same conditions were lysed for detecting total protein with BCA reagent.

**Cell apoptosis analysis.** Flow cytometric analysis using PI was performed to determine the apoptosis. Cells that were less intensely stained than G1 cells (sub G0/G1 cells) in flow cytometric histograms were considered apoptotic cells. Briefly, treated INS-1 832/13 β-cells (1×10⁶) were collected by trypsinization after the corresponding experimental periods, washed twice with PBS, and fixed in ice cold 70% ethanol at 4°C overnight. After being washed twice with cold PBS, cell pellets were resuspended in PBS with 40 µg/mL RNase for 30 min at 37°C, followed by incubation in PBS containing 40 µg/mL PI and stood in the dark for 15 min at room temperature before being analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). DNA content (2X104 cells) was analyzed for each sample using CellQuest software and data were consequently evaluated by Cell Quest (Becton Dickinson) and ModFit LT software. The experiment was performed as triplicate independent experiments.

**Determination of intracellular reactive oxygen species (ROS).** INS-1 832/13 β-cells were cultured in 96-well black plates with 200 µL of growth medium and incubated for 24 h at 37°C. At the end of treatment the

![Fig. 1. Cytotoxicity of various concentrations of Vc (A) and its protective effect on cell viability of INS-1 832/13 cells with glucolipotoxicity (B). *p<0.05. The cells were firstly treated with different concentrations of Vc in DMEM medium for 48 h (A). In figure B, control cells were treated with 0.5% BSA in 5.6 mM glucose medium, and model cells were treated with different concentrations of Vc in 22.4 mM glucose plus 0.5 mM FFA for 36 h.](image)

**Table 1. Effect of Vc on glucolipotoxicity-induced TG, apoptosis and ROS increase.**

| Group      | TG (nmol/mg Pro) | Apoptosis (%) | ROS (%)   |
|------------|------------------|---------------|-----------|
| Control    | 23±3             | 1.59±0.26     | 32.5±6.6  |
| Model      | 154±9*           | 9.93±0.41*    | 100.0±15.1* |
| Model+1 µg/mL Vc | 138±14*  | 8.97±0.48     | 66.1±5.9*  |
| Model+10 µg/mL Vc | 138±17*  | 5.68±0.22*    | 70.0±2.7*  |
| Model+50 µg/mL Vc | 122±12*  | 4.14±0.44*    | 42.9±10.1*  |

* p<0.05, compared to model; # p<0.05, compared to control.
medium was removed and cells were washed with PBS. Then 25 μM DCFH-DA was added and incubated for 30 min at 37°C to allow cell uptake. Within the cells, DCFH-DA was hydrolyzed to dichlorodihydrofluorescin (DCFH) and the DCFH emitted fluorescence when it was oxidized to dichlorofluorescin (DCF). Therefore, the fluorescence in the cells directly reflected the level of ROS. After incubation with DCFH-DA, the cells were washed thoroughly with HBSS and then 50 μL HBSS was dispensed into each well. The fluorescence of the cells from each well was recorded by the microplate reader at 530 nm emission and 485 nm excitation.

For cocultured β-cells, cells were washed with PBS and incubated with 25 μM DCFH-DA for 30 min at 37°C. The cell pellets were washed twice with HBSS, and the fluorescence was detected using a FACS Calibur flow cytometer at 525 nm emission and 488 nm excitation.

Determination of intracellular TG. The cells were washed twice with pre-cooled PBS after treatment for 24 h, followed by an addition of RIPA lysis buffer. The cell lysates were centrifuged at 10,000 rpm for 5 min at 4°C. Intracellular TG in collected supernatants was determined using a TG kit.

Statistical analysis. Data were expressed as the mean values ± standard deviation (SD) for each measurement, analyzed by one-way analysis of variance (one-way ANOVA) followed by Tukey’s post hoc test (SPSS 13.0). Results were considered significant at a p-value of 0.05.

RESULTS

Effect of Vc on cell viability, apoptosis and ROS production of glucolipotoxicity-induced β-cells

To avoid cell death caused by Vc, the viability was determined after Vc incubation for 36 h by MTT assay. As shown in Fig. 1A, the concentrations of Vc at less than 50 μg/mL did not affect cell viability (p > 0.05). After 36 h treatment with high levels of glucose and FFA, the cell mass dramatically dropped to 70% of that of the control group (p < 0.05), whereas the addition of Vc increased cell viability in a dose-dependent manner (Fig. 1B). Compared to the model group, cell viability was significantly (p < 0.05) increased about 33% by Vc at the concentration of 50 μg/mL. As expected, the number of apoptotic cells was approximately 6 times that of the control (Table 1), and the treatment with 10 μg/mL and 50 μg/mL Vc reduced apoptosis by about 42% and 59%, compared to the model group, respectively. In the meantime, cellular ROS of the model group was significantly elevated nearly 3-fold over the control group (p < 0.05). At concentrations of 1, 10 and 50 μg/mL, Vc significantly scavenged ROS by about 34%, 30% and 57%, respectively (p < 0.05 vs. Model).

Effect of Vc on TG accumulation, insulin secretion function and intracellular insulin content of glucolipotoxicity-induced β-cells

Thirty-six hours exposure to high levels of glucose and FFA led to a significant increase in TG accumulation by INS-1 832/13 cells compared to the control (p < 0.05; 22.7 ± 2.8 vs. 153.7 ± 9.2; Table 1). This alteration was significantly ameliorated by Vc treatment. At 50 μg/mL, Vc inhibited nearly 21% TG deposition (p < 0.05 vs. Model). In addition, the value of GSIS was significantly decreased from 323 to 261 μU/mg Pro-h after long-term incubation with high levels of glucose and FFA, and BIS was provoked 1.5 fold compared to the control (Fig. 2A; p < 0.05). Though Vc mildly raised GSIS, the
improvement did not reach statistical significance. The main action of Vc was to reduce the elevated BIS, resulting in significantly higher ISI indices (Fig. 2B; \( p<0.05 \)). However, Vc supplement did not significantly alter the reduced intracellular insulin level caused by glucolipotoxicity (Fig. 2C; \( p>0.05 \)).

**Effect of Vc on TG deposition in adipocytes and β-cells and levels of fatty acids in the medium of the coculture system**

The cell viability of β-cells was not changed during the 48 h coculture experiment (Data not shown). As shown from Fig. 3, after exposing β-cells to the system containing fully differentiated 3T3-L1 adipocytes, elevated TG content was found in both β-cells and adipocytes at 1.8-fold and 1.3-fold, respectively (\( p<0.05 \), compared to relevant control). The increased TG content estimated in β-cells was remarkably reduced by the addition of 10 and 50 μg/mL Vc, whereas 50 μg/mL Vc also effectively reduced the extent of TG content increase in adipocytes when compared to the cocultured adipocytes. Meanwhile, the level of fatty acids was dramatically increased (\( p<0.05 \)) in the coculture medium, and the addition of 50 μg/mL Vc significantly (\( p<0.05 \)) reduced fatty acids by approximately 36% compared to cocultured model system (Fig. 3C).

**Intracellular insulin content and insulin secretion function of β-cells in the coculture system**

When β-cells were cocultured with adipocyte but not pre-adipocyte, ROS of the β-cells was significantly increased (\( p<0.05 \)) compared to the control, as seen from Fig. 4A. Vc treatments markedly (\( p<0.05 \)) reduced the ROS level compared to cocultured β-cells. Additionally, insulin secretion functions of β-cells were dramatically changed, as seen from Fig. 4B. BIS of the coculture group was about twice that of the control group, and GSIS was reduced to 57% of that of the control group. The presence of 10 and 50 μg/mL Vc in the coculture system did not affect BIS, but effectively raised GSIS by 50% and 60%, respectively (\( p<0.05 \), compared to cocultured β-cells). Improvements in insulin secretion led to recovered ISI indices in both the 10 and 50 μg/mL Vc treatments (Fig. 4C). However, the addition of Vc did not increase the reduction in intracellular insulin of β-cells (Fig. 4D; \( p>0.05 \)).

**IL-6 and adiponectin levels in the coculture system**

In the present study, IL-6 and adiponectin, two secretory factors released from adipocytes to the medium of the coculture system, were detected by Elisa kits. Figure 4E and 4F show the changes of these two factors. After being cocultured with β-cells, adipocytes secreted more IL-6 than that of the control group by 43%, along with an increase in the adiponectin level by 7-fold compared to that of the control group. Vc at different doses significantly reduced IL-6 secretion (\( p<0.05 \), compared to the cocultured group), but its efficacy was weakened with the concentration. The adiponectin level, however, did not show obvious changes in the presence of Vc.

**DISCUSSION**

Fatty acids and glucose are important physiological fuel for pancreas islets, and act as secretagogues to insulin release. However, prolonged exposure of β-cells to excess levels of these nutrients leads to β-cell secretion dysfunction and cell death induced by apoptotic progress (5). In the present study, after 36 h incubation with high levels of glucose and FFA, INS-1 832/13 β-cells demonstrated significant reduction in cell mass and significant increase in apoptotic cells and ROS levels (\( p<0.05 \)). Considerable evidence suggests that chronic elevation of glucose and FFA leads to increased oxidative stress through increased generation of ROS, thereby mediating apoptosis-related genes and caspase-3 to induce apoptosis in β-cells (21, 22). Given the strong
antioxidant activity of Vc and its potential to elevate intracellular antioxidants (23), the ROS generation and subsequent apoptosis suppressed by Vc at the concentration of 50 μg/mL (Table 1; p<0.05) was expected and suggests that Vc can enhance cell viability against glucolipotoxicity.

Besides inhibition of cell viability, glucolipotoxicity also damaged β-cell secretion function. When treated with high levels of glucose and FFA for 36 h, BIS was elevated along with decreased GSIS (Fig. 2A). Data on altered insulin secretion function implied that β-cells were under compensation status in secreting more insulin at low glucose concentration, thereby causing a decrease in intracellular insulin stores. In addition, the reduction in GSIS suggested β-cells lost their sensitivity to high glucose. This result is consistent with a previous report when rat pancreas islets were exposed to the culture medium with different fatty acids (24). Glucolipotoxicity-induced oxidative stress is reported to depress the expression of pancreatic and duodenal homeobox factor-1 (PDX-1), which is a β-cell-specific transcription factor (25). Additionally, excess TG deposition in β-cells could affect energy metabolism and impair insulin secretion (26). The presence of Vc effectively reduced TG deposit in β-cell under both glucolipotoxicity and coculture conditions (Table 1 and Fig. 3A; p<0.05). Therefore, in a glucolipotoxicity or adipocyte coculture situation, Vc at a certain concentration might ameliorate the BIS increase or GSIS decrease by scavenging intracellular ROS and TG. In vivo, the combination of N-acetyl-L-cysteine (NAC), Vc and vitamin E preserves the insulin content and insulin mRNA of T2DM mice (27). Additionally, the Vc and vitamin E combination could regulate PDX-1 to ameliorate β-cell dysfunction (27). Therefore, in the present study, Vc might have acted in the same pathway to preserve β-cell secretion function under both glucolipotoxicity and coculture conditions. However, Vc was ineffective in recovering intracellular
insulin content. The possible related mechanism for such confliction deserves further investigation.

In the coculture system, TG in adipocytes was decreased when 50 µg/mL Vc was added, associated with less FFA release into the medium. As it is known, adipocytes would release a large amount of FFA as well; thus, improvement in β-cell dysfunction by Vc addition in the coculture system might be through the same mechanism of weakening glucolipotoxicity. In a recent study, coculturing adipocytes is reported to have an adverse impact on β-cell function, such as reduction in intracellular calcium concentration and loss of the insulin secretion response to tolbutamide (14). Moreover, inhibition of insulin, glucokinase and Kir6.2 genes is noted in a 3T3-L1 adipocyte coculture study (14). However, the specific secretion factor which directly damages β-cells in the coculture system remains unclear. IL-6 and adiponectin, however, are known to increase insulin secretion and preproinsulin mRNA expression (10, 28). Based on the beneficial effect of IL-6 and adiponectin, the levels of these two factors were determined in the current study. It was found that IL-6, but not adiponectin, increased in a dose-dependent manner. However, the IL-6 level was still lower than that of the cocultured cells (Fig. 4E and 4F). Therefore, there might be other factors associated with Vc treatment and further interaction with β-cells.

In the present study, ISI indices for the control group were different in glucolipotoxicity and the coculture system. For glucolipotoxicity treatment, β-cells were incubated in the medium containing 5.6 mM glucose and 0.5% BSA, whereas in the coculture system, the medium was the mixture of two complete media containing 10% FBS. Thus the presence of FBS might be partially responsible for the elevation in ISI (3.81 vs. 2.16; Fig. 2B and Fig. 4B). The mechanisms underlying the beneficial effect of Vc on the relationship of β-cell dysfunction needs further investigation.

CONCLUSION

In this study, we have found that antioxidant treatment using Vc can improve cell viability by reducing apoptosis and ROS levels through reduction in glucolipotoxicity, preservation of secretion function and inhibition of TG accumulation in β-cells induced by glucolipotoxicity and adipocyte coculture. The positive effects of Vc might be associated with amelioration effects on ROS generation and intracellular TG level regulation by Vc addition at certain concentrations.

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