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EGCG and quercetin protected INS-1 cells in oxidative stress via different mechanisms

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1. ABSTRACT

EGCG and quercetin are known as beneficial dietary flavonoid for various diseases including diabetes mellitus. But it is not certain whether they could protect pancreatic beta cell directly. We performed this study to test both EGCG and quercetin could directly protect beta cell line under oxidative stress, and verify the action mechanisms. The protective effect of quercetin on INS-1 cells against oxidative stress was concentration dependent, but EGCG showed specific concentration zone for the protection. The protective effect of EGCG was more pronounced in pre-treatment before oxidative stress, while quercetin showed dramatic improvement of viability in simultaneous incubation with H_2O_2. In EGCG pre-treatment, antioxidant enzymes and activity were decreased, but the phosphorylated PI3K and Akt were significantly increased. PI3K inhibitor significantly reduced cell viability in EGCG pre-treatment. In conclusion, EGCG and quercetin have protective effect on INS-1 cells against oxidative stress through both antioxidant effect and anti-apoptosis signaling. In EGCG, pre-treatment make its effect better by the enhancement of anti-apoptosis signaling. Quercetin protected INS-1 cells more in simultaneous incubation via strong antioxidant defense.

2. INTRODUCTION

Beta cells in diabetics could be damaged by various cytotoxic stimuli including chronic exposure to high glucose and fatty acid. Oxidative stress is suggested as a final common link (1-6). The insulin secreting beta cells are known to be vulnerable to oxidative damage with an increased sensitivity for apoptosis. This is due to excessive mitochondrial ROS (reactive oxygen species) generation, additional ROS generation through elevated beta-cell NADPH oxidase activity, and relative lack of antioxidant defense system (7-11). Several studies have shown that ROS could regulate the activation of Akt, a downstream effector of PI3K, in cultured cells such as fibroblasts, mesangial cells, and vascular smooth muscle cells (12,13). It has also been reported that the activation of PI3K/Akt signaling during the cellular response to oxidant injury was important for survival in various diseases including diabetes (14,15).

Epigallocathechin gallate (EGCG) and quercetin are two of the most frequently studied dietary flavonoids. EGCG is one of the major beneficial ingredients of green tea, the most popular beverage in the world. Quercetin is

plentiful in various vegetables and fruits, especially grape (16,17). EGCG and quercetin have both pro-apoptotic and anti-apoptotic effects on different cell types. While they could increase apoptosis in the various cancer cell lines, their anti-apoptotic efficacy in cardiovascular, skin and neurodegenerative disease has been reported (18-27). The underlying mechanisms of both sides of actions are still remained unclear. In addition to their classical antioxidant effect, recent studies have speculated that EGCG and quercetin might have modulatory effects onto cells through several protein kinase and lipid kinase signaling pathways (28-31).

Flavonoids have been proposed to exert beneficial effects in various diseases, including cancer, cardiovascular disease and neurodegenerative disorders (31). The amounts of EGCG and quercetin consumption have shown inverse relationship with the prevalence of diabetes mellitus in human (32,33). The antihyperglycemic effect of EGCG and quercetin was come from their effects to increase glucose tolerance, stimulate insulin secretion, increase insulin stimulated glucose uptake, inhibit intestinal glucose transporter and decrease the expression of genes that control gluconeogenesis (34-39). Flavonoids may preserve ß-cell function by reducing oxidative stress-induced tissue damage, however, the relation of dietary flavonoids to the pancreatic beta cell death is less well studied (33).

The clinical importance of identifying substances to protect beta cells against oxidative stress and the ability of EGCG and quercetin to increase cell survival in other cell lines through different mechanisms led us to investigate the effect of both flavonoids on cell death induced by oxidative stress and their mechanisms in insulin-producing INS-1 cells.

3. MATERIALS AND METHODS

3.1. Chemicals

EGCG, quercetin, RPMI 1640 medium, and H_2O_2 were purchased from Sigma-Aldrich (MO, USA) and wortmannin was obtained from Cell signaling (MA, USA). Glucose, HEPES, and MTT were purchased from Amresco (OH, USA). Annexin V and propidium iodide (PI) were obtained from BD bioscience (CA, USA). Glutathione peroxidase assay kit and superoxide dismutase assay kit was bought from Cayman Chemical Company (MI, USA).

3.2. Cell culture

INS-1 rat insulinoma cell lines were obtained from ATCC (Manassas, VA). INS-1 cells between 30^{th} and 40^{th} passages were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 10mM HEPES, 11mM Glucose, and 50μM 2-mercaptoethanol at 37°C with 5% CO_2

3.3. Cell viability

INS-1 cells were seeded in 96-well plates at 7×10^3 cells per well and incubated for 48 hr. To evaluate the oxidative stress by H_2O_2 and protective effect of EGCG or quercetin, mediums were aspirated and new mediums containing various concentrations of H_2O_2 and/or EGCG or quercetin were added and further incubated for additional 5 hr. Pre-incubation experiments were done by adding EGCG or quercetin 24 hr prior to the addition of H_2O_2. To use PI3K inhibitor, wortmannin, cells were pre-incubated for 1hr prior H_2O_2 and the addition of EGCG or quercetin. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The optical density was measured at 570 nm by automatic plate reader.

3.4. Annexin V staining for the detection of apoptosis

INS-1 cells were plated in 6-well plates at 5×10^4 cells per well and incubated in appropriate condition. To make single cells, cells were treated with trypsin-EDTA and centrifuged 1500rpm for 5min at 4°C. After aspirating supernatants, cells were washed with 1 ml of Annexin V binding solution (140mM NaCl, 10mM HEPES pH 7.4, 2.5mM $CaCl_2$) and centrifuged 1500rpm for 5min at 4oC. Supernatants were removed and 3 μl of Annexin V-FITC and 10μl of propidium iodide were added. After incubation for 15 min in the dark, 300μl of FACS buffer (1% FBS, 0.1% NaN_3) were added and analyzed by FACSort (BECTON DICKINSON, BD bioscience).

3.5. Activity of glutathione peroxidase and superoxide dismutase

INS-1 cells were plated in 6-well plates at a density of $5x10^4$ cells per well, and incubated for at least 48hr to reach 60-70% confluence. The incubation condition was carried out as mentioned above. After finishing incubation, the cells were rinsed with PBS and lysed with mammalian tissue Lysis/Exraction reagent (Sigma, MO, USA). The activity of glutathione peroxidase and superoxide dismutase were measured according to the manufacturer's instructions.

3.6. Reverse Transcription Polymerase Chain Reaction

Total cellular RNA was isolated using Trizol reagent (Invitrogen, CA, USA). RT-PCR was performed using premix RT-PCR kit (Bioneer, Daejeon, Korea). Amplification was carried out under the following conditions using MyCycler thermal cycler (Bio-rad, CA, USA): pre-denaturation 95°C for 2min, denaturation 95°C for 30s; annealing 48°C for 30s; extension 68°C for 30s, final extension 68°C for 7min. After amplification, 2μl of PCR products were subjected to electrophoresis on 1.5% agarose gels with ethidium bromide. The gels were visualized by SL-20 DNA Image Visualizer and quantified by GelDoc densitometry.

3.7. Western blot

INS-1 cells were washed with PBS and lysed with mammalian tissue Lysis/Exraction reagent including protease inhibitor. The amount of total protein was quantified by BCA protein assay kit, and 1× SDS sample buffer(50mM Tris pH6.8, 2% SDS, 10% glycerol, 50mM DTT, and 0.01% bromophenol blue) was added to the extracted protein. Proteins were separated at 12% SDS-PAGE, transferred on PVDF membrane, and immunoblotted with anti-phospho p85 PI3K (Tyr 458) (1:1000), anti-total Akt (1:1000), anti-phospho Akt (Ser

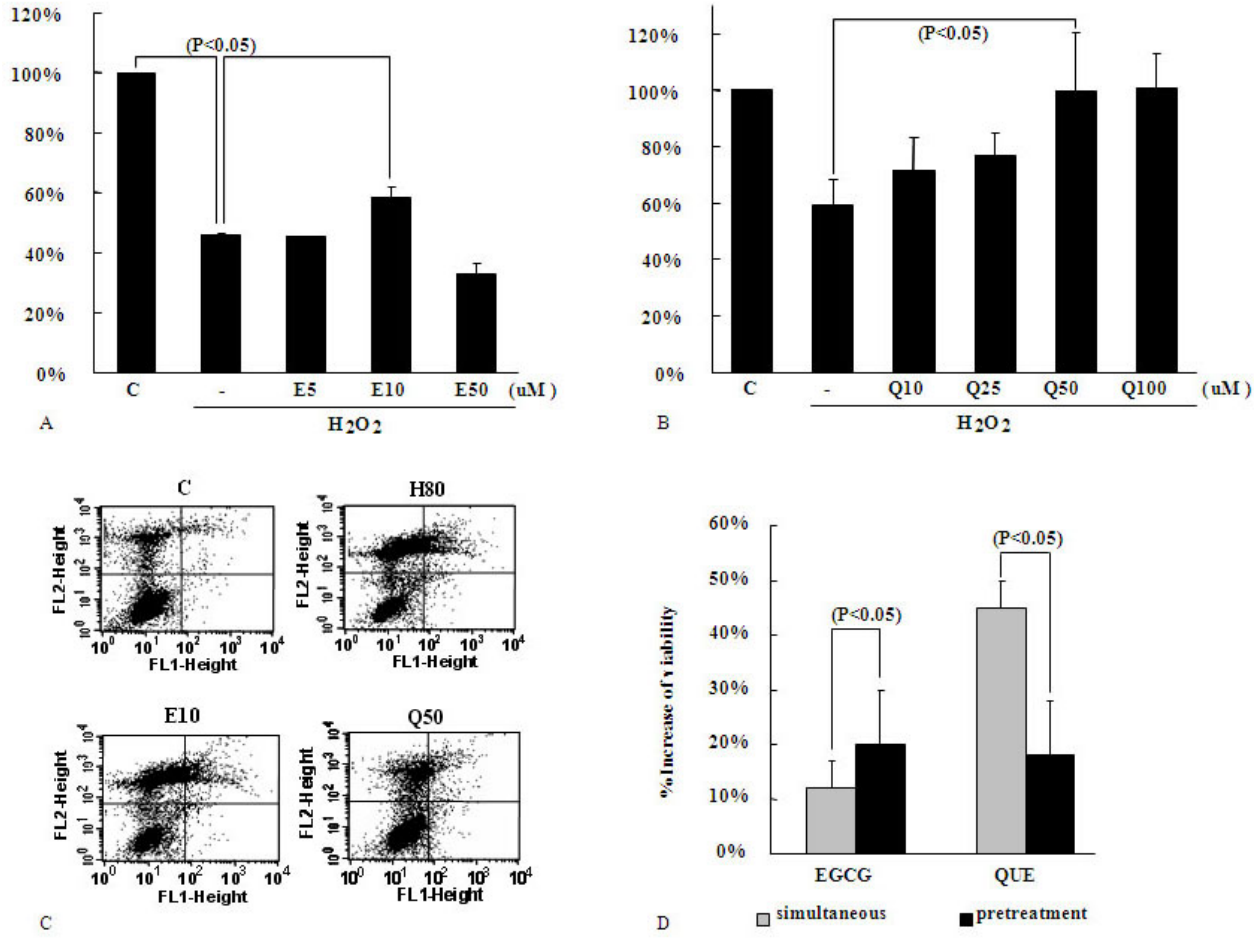

Figure 1. Effect of EGCG and Quercetin on INS cell viability. (A) The viability was assessed under H_2O_2 with or without various concentrations of EGCG by MTT assay. (B) The viability was assessed under H_2O_2 with or without various concentrations of Quercetin by MTT assay. (C) The viability was assessed without H_2O_2 (C), with H_2O_2 (H80), H_2O_2 + EGCG 10μM (E10) and H_2O_2 + Quercetin 50μM (Q50) by FACS. (D) The increase of viability was compared to only H_2O_2 treated group in simultaneous apply of H_2O_2 and EGCG or Quercetin and EGCG or Quercetin pre-treatment before H_2O_2 apply, respectively.

473) (1:1000), anti-caspase 3 (1:1000), anti-Mn SOD (1:2000), and anti-catalase (1:2000) at 4°C for overnight.

Secondary antibodies as goat anti-rabbit conjugated alkaline phosphase were applied for 1hr at room temperature and membrane was developed by AP-conjugated development kit (Bio-rad, CA, USA). Developed protein bands were quantified by Multi Gauge V2.2 program.

3.8. Measurement of secreted insulin by ELISA

After incubation, cells were washed with PBS. To test glucose-stimulated insulin secretion(GSIS), cells were starved for 5hr in RPMI medium containing 5mM glucose and 2% FBS. Then medium were changed to KRBB solution (4.74mM KCl, 1.19mM KH_2PO_4, 1.19mM $MgCl_2 \cdot 6H_2O$, 35mM $NaHCO_3$, 10mM HEPES) containing 5mM glucose or 25mM glucose and cells were incubated for additional 1hr. Secreted insulin in the medium was measured using Rat/Mouse Insulin ELISA kit (Linco research, MO, USA).

4. RESULTS

4.1. Protection of INS-1 cells from oxidative stress by EGCG and quercetin is dependent on the concentration and the incubation time

In order to find the LD50 for hydrogen peroxide in INS-1 cells, we tested 0, 20, 50, 80, and 100μM hydrogen peroxide for 5hrs, and it was 80μM hydrogen peroxide (data not shown). When flavonoids and H_2O_2 were treated simultaneously, EGCG increased INS-1 cell viability at 10μM. But the viability was decreased over 50μM of EGCG (Figure 1A). In the case of Quercetin, it increased INS-1 cell viability depending on its concentration till 50μM, and then kept plateau to 100μM (Figure 1B). We re-confirmed INS-1 cell viability using the most effective concentrations of EGCG and Quercetin by FACS, and the results were similar to MTT assay (Figure 1C).

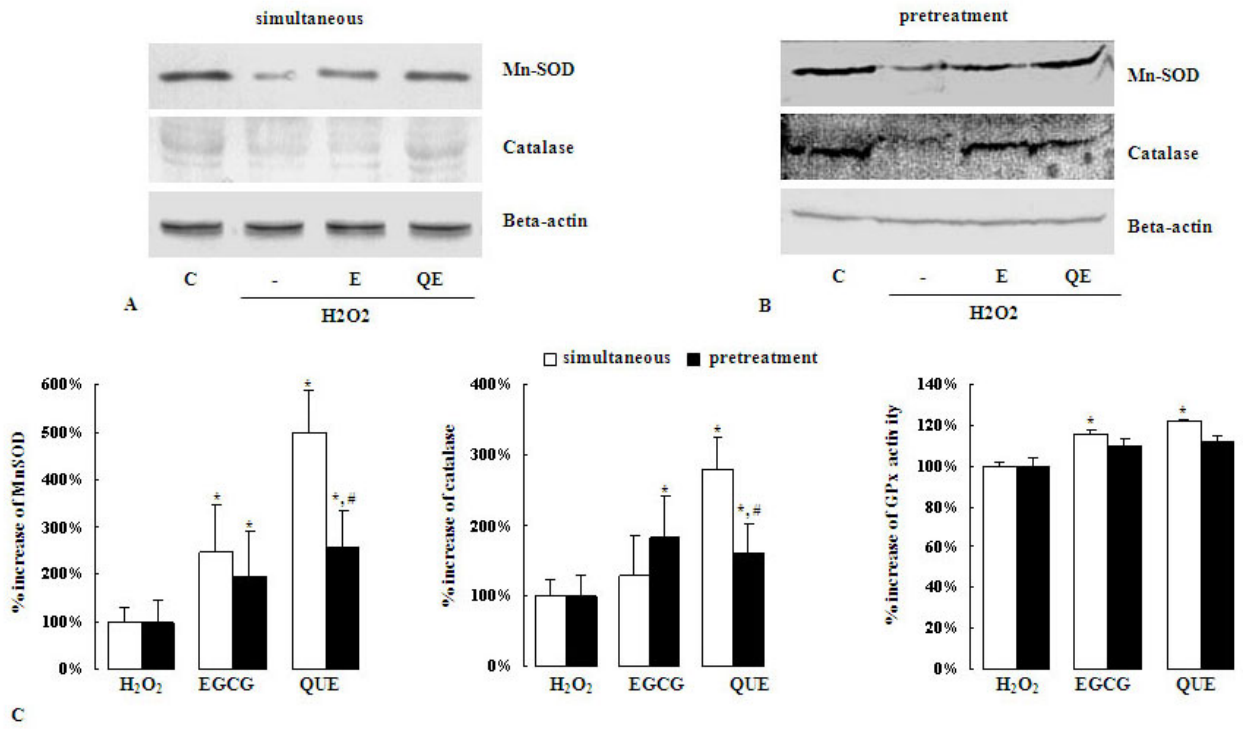

Figure 2. The changes of MnSOD, catalase and GPX activity with EGCG or Quercetin treatment. (A) H_2O_2 with EGCG or Quercetin applied simultaneously and the changes of MnSOD and catalase were assessed by western. (B) EGCG or Quercetin applied before H_2O_2 and the changes of MnSOD and catalase were assessed by western. (C) The percent increase of MnSOD compared with H_2O_2 only group depending on EGCG and Quercetin in simultaneous apply and pretreatment method. (D) The percent increase of Catalase compared with H_2O_2 only group depending on EGCG and Quercetin in simultaneous apply and pretreatment method. (E) The percent increase of MnSOD compared with H_2O_2 only group depending on EGCG and Quercetin in simultaneous apply and pretreatment method. *: p value < 0.05 compared to H, #: p value < 0.05 compared to simultaneous

We compared the cell protective effect of each substance with different incubation protocols. Quercetin was stronger than EGCG in decreasing cell death in simultaneous treatment, but its effect was comparable to EGCG in pretreatment. When we pre-treated flavonoids 24 hours before H_2O_2, EGCG protected INS-1 cell death more effectively than simultaneous administration, while the protective effect of quercetin was significantly decreased in pre-treatment (Figure 1D).

4.2. EGCG and Quercetin changed antioxidant enzyme system

Both EGCG and Quercetin showed statistically significant increase of MnSOD protein compared to only H_2O_2 treatment group ($p<0.05$) (Figure 2). But in pre-treatment protocols, the increases were significantly lower than simultaneous treatment, especially in Quercetin. In case of catalase, quercetin significantly increased its protein amount ($p<0.05$), but not in EGCG in simultaneous treatment. Both flavonoids increased catalase protein in pretreatment experiment, but the amount of increase in quercetin was significantly smaller than simultaneous treatment (Figure 2). When we applied EGCG 10μM or Quercetin 50μM with H_2O_2 simultaneously, glutathione peroxidase activity was increased in both EGCG and Quercetin treatment group compared to only H_2O_2 treatment and the amount of increase was higher than pretreatment of EGCG and Quercetin (Figure 2).

4.3. EGCG and quercetin modulated antiapoptosis signaling

EGCG didn't increased PI3K phosphorylation and Akt total amount and phosphorylation at simultaneous treatment with H_2O_2, but showed significant increase in pretreatment (Figure 3). In case of quercetin, increased PI3K and Akt proteins significantly at both simultaneous and pretreatment (Figure 3). The increase in Akt phosphorylation in EGCG under pretreatment was higher than quercetin group. Comparing H_2O_2 only treatment, EGCG didn't show any change in caspase 3 protein in western immoblot in simultaneous treatment while it increased caspase 3 in pretreatment. Quercetin showed significant increase of that in both conditions of simultaneous and pretreatment.

4.4. The effect of EGCG and quercetin for protection against oxidative stress was reduced partially by Wortmannin and those were different by incubation time in each flavonoid

The % inhibition of protection was calculated by the way described in Figure 4. The protective effect of

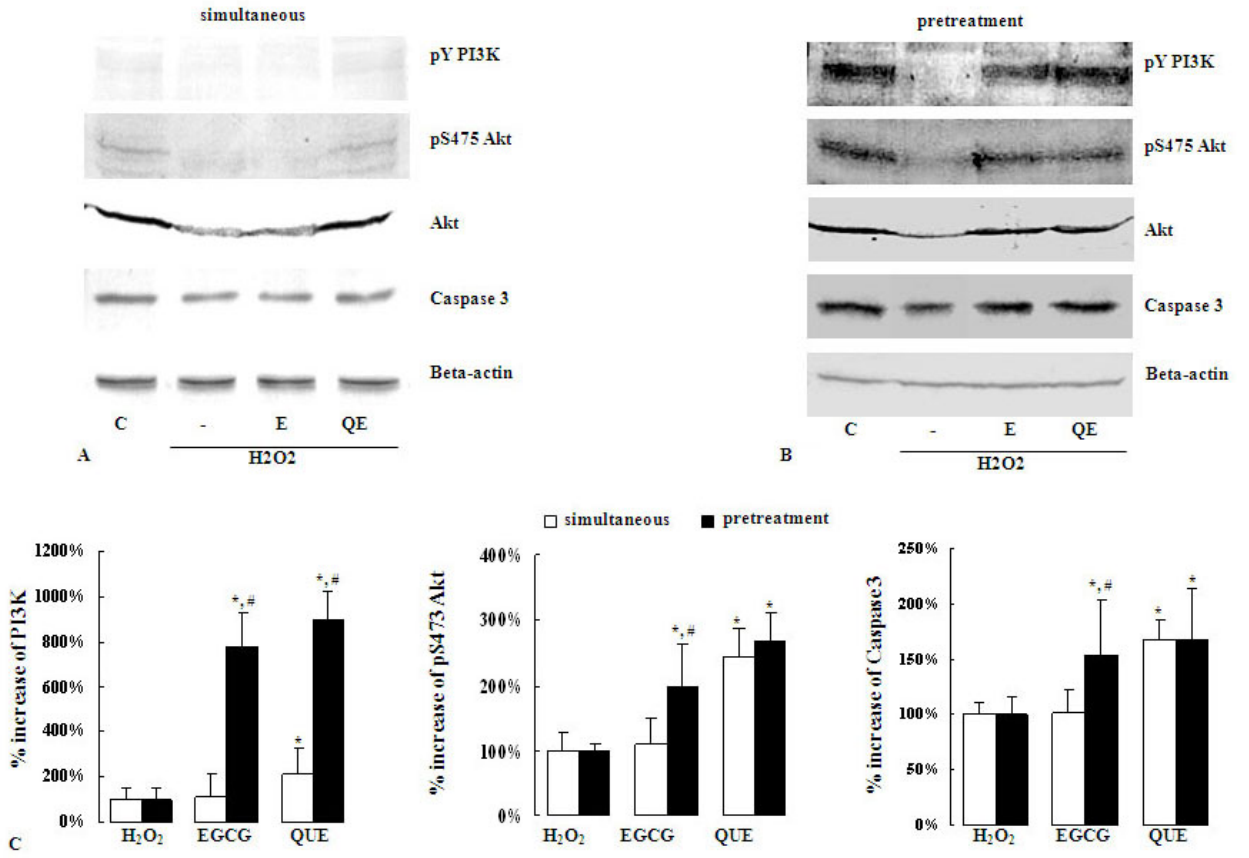

Figure 3. The changes of PI3K, Akt and caspase 3 with EGCG or Quercetin treatment. (A) H_2O_2 with EGCG or Quercetin applied simultaneously and the changes of PI3K, Akt and caspase 3 were assessed by western. (B) EGCG or Quercetin applied before H_2O_2 and the changes of PI3K, Akt and caspase 3 were assessed by western. (C) The percent increase of PI3K compared with H_2O_2 only group depending on EGCG and Quercetin in simultaneous apply and pretreatment method. (D) The percent increase of pS473Akt compared with H_2O_2 only group depending on EGCG and Quercetin in simultaneous apply and pretrement method. (E) The percent increase of caspase 3 compared with H_2O_2 only group depending on EGCG and Quercetin in simultaneous apply and pretreatment method. *: p value < 0.05 compared to H, # : p value < 0.05 compared to simultaneous

EGCG was decreased by 50% with PI3K inhibitor in pretreatment, which is five fold higher compared to simultaneous treatment. In terms of quercetin, the effect of PI3K inhibitor was larger in simultaneous treatment than pretreatment, which was smaller than in EGCG under pretreatment.

4.5. EGCG and quercetin restored Glucose stimulated insulin secretion

In order to check INS cell function, we tested insulin secretion at the concentrations of 5mM and 25mM of glucose. At 5mM glucose concentration, EGCG and quercetin restored insulin secretion significantly, and the amount of increase was higher in quercetin group. At 25mM glucose, they also increased insulin secreation. The amount of GSIS from 5mM to 25mM of glucose was significantly higher in EGCG and quercetin in both simultaneous and pretreatment condition (Figure 5).

5. DISCUSSION

Dietary flavonoids, EGCG and quercetin have been reported that they have dual effects in apoptosis of cell and their beneficial and deleterious effects were dependent on cell types and their own concentrations. In tumor cells, both flavonoids induced apoptosis (18-20,23,24). Quercetin prevent apoptosis in several cell lines such as fibroblasts, cardiomyoblast cells, and epithelial cells (21,22) and EGCG also increased viability in neural cell lines, skin cells and cardiomyocytes (18-20). EGCG can induce apoptosis in high concentration more than 50μM of EGCG and has opposite effect in lower concentration (25,26). Quercetin at the doses below 100 μM significantly decreases H_2O_2-induced peroxide production, however quercetin induction of apoptosis is detected at the dose of 200 μM (34). H_2O_2-induced DNA damage was inhibited with 10 μM of either EGCG or quercetin in Jurkat T-lymphocytes. In contrast, significant DNA damage was induced by incubating with 10-fold higher concentrations, 100 μM of either EGCG or quercetin by themselves (27). Those concentrations of both flavonoids for antiapoptosis and proapotosis were different on various cells. In our study, EGCG and quercetin inhibited INS cell death and restored insulin secretion

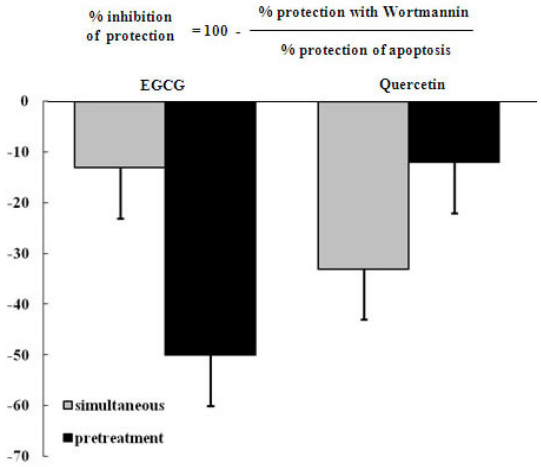

Figure 4. The percent inhibition of protection after apply of PI3K inhibitor. The changes of apoptosis of INS cell were assessed when PI3K applied before EGCG or Quercetin treatment in simultaneous and pre-treatment condition, respectively. The percent inhibition of protection compared with EGCG or Quercetin protection against H_2O_2 was calculated by the equation suggested here..

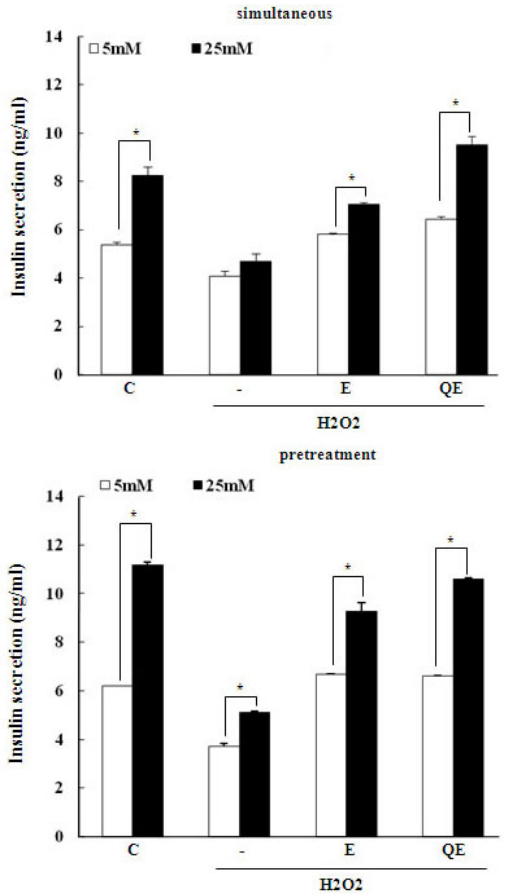

Figure 5. The Glucose stimulated insulin secretion (GSIS) with EGCG or Quercetin treatment in simultaneous and pretreatment condition, respectively.

under oxidative stress. 10 μM of EGCG increased cell viability but over 50 μM of that increased cell death. Quercetin increased cell viability as increasing its concentration till 100 μM in INS cell.

The reason why EGCG and quercetin have different effect on apoptosis may be explained by tissue specificity and concentration as shown above. In addition to those, incubation time can also change their effect. In terms of incubation time, it was various depending on the tissue, the amount and sort of oxidative stress and concentration of flavonoids (27, 35, 36). They also used different applying method like oxidative stress and flavonoid treat simultaneously or pre-treat flavonoid and then stress. In our experiment, EGCG increased cell viability more in pretreatment, but in case of quercetin, it was better in simultaneous way in INS cell.

The protective effects of EGCG and quercetin have been identified in different cells, however the mechanism is still unclear . Traditionally, EGCG and quercetin are well known as stronger anti-oxidants than Vitamin C and Vitamin E (37). Pancreatic beta cells which are readily destroyed by oxidants, and in which there is unusually low expression of antioxidant enzymes, particularly of catalase and Gpx (10, 11). Overexpression of catalase in beta cell line showed excellent protection against the toxicity of extracellular H_2O_2. In contrast, Gpx showed only a modest protective effect. Although cells transfected with SOD alone were not protected from H_2O_2, a combination of SOD and catalase gave much better protection than catalase alone, reflecting the ability of SOD to maintain catalase in its active form (8). In present study, quercetin significantly increased SOD and catalase in simultaneous treatment but decreased to comparable to EGCG in pretreatment, while Gpx activity was similar in two flavonoids treatment under both incubation condition and those results were matched well with the results of viability. According to previous study, our results may explain that quercetin protected INS cell more than EGCG in simultaneous treatment through antioxidant enzyme activation, especially MnSOD plus catalase.

Flavonoids, in addition to the classical antioxidant capacity, have been reported to exert modulatory effects in cells through selective actions at different components of protein kinase and lipid kinase signaling cascades (39). PI3K/Akt and GSK-3 activities are altered in G93A mutant cells and EGCG-induced activation of PI3K/Akt and inhibition of GSK-3 could be a new potential therapeutic strategy for ALS associated with oxidative injury (40). EGCG has neuroprotective effect by increasing PI3K/Akt-dependent anti-apoptotic signals (41), stimulation of PKC and a modulation of cell survival/cell cycle genes, such as Bax, Bad, Mdm2, Bcl-2, Bcl-w, and Bcl-x(L) (42). Quercetin was able to induce apoptosis in tumor cells through activation of caspase 3 cascades, suppression of heat shock protein 70 (23, 24) and intervention in the JNK and ERK-mediated apoptotic pathways (43). This present study showed PI3K/ Akt activities were increased in pre-treatment

compared to simultaneous treatment in both flavonoids treatment. The amounts of increase in phosphorylted PI3K and Akt were higher in EGCG than quercetin when we compared pre-treatment to simultaneous treatment. The percent inhibition of protection in viability after treatment of wormannin was also higher in EGCG than quercetin in pre-treatment. Those results may mean that the protective effect of EGCG is more dependent on anti-apoptosis mechanism than antioxidant and that effect need some incubation time. Some reported flavonoids need an incubation time to modulate cell signaling. A pre-incubation of cells with EGCG for at least 30 minutes, or 8 to 16 hours, was employed to show its modulatory effect on EGFR phosphorylation (44, 45). Similar pre-incubation periods were used to observe an inhibitory effect of EGCG on PDGFRß phosphorylation (46, 47). Contrast to anti-apoptosis system, antioxidant system was deteriorated under pre-treatment with EGCG and quercetin compared to simultaneous treatment. The relationship between oxidation stress and phosphoinositide 3-kinase (PI3K) signaling in pancreatic ß-cell dysfunction remains unclear (15).

In our study, we saw that EGCG and quercetin could protect INS cell as in other cell lines and restored insulin secretion against oxidative stress. They seem to exert their effects via dual actions on the antioxidant defense and anti-apoptosis pathway. The protective effect of EGCG was stronger in pre-treatment by the better enhancement of anti-apoptosis mechanism while quercetin protected INS cell significantly in simultaneous treatment and it was more dependent on the activation of antioxidant defense. Further studies will be necessary for more detailed action mechanisms and the clinical implication.

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