Recovery Effect of Onion Peel Extract against H$_2$O$_2$-Induced Inhibition of Gap-Junctional Intercellular Communication is Mediated through Quercetin

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Abstract: Cellular oxidative damage mediated by reactive oxygen species has been reported to inhibit gap–junctional intercellular communication (GJIC). In turn, the inhibition of GJIC can be attenuated by functional food compounds with antioxidant properties. In this study, we compared the protective effects of onion peel extract (OPE) and onion flesh extract (OFE) on oxidative stress-mediated GJIC inhibition, and investigated the mechanisms of action responsible. OPE restored H$_2$O$_2$–induced GJIC inhibition to a higher degree than OFE in WB-F344 rat liver epithelial cells. OPE was found to inhibit H$_2$O$_2$–induced phosphorylation of ERK1/2 and Cx43. A radical scavenging assay demonstrated superiority of OPE over OFE, suggesting that the observed effects might be mediated via an antioxidant mechanism. Quercetin is the major compound that is likely to be responsible for the protective effect against H$_2$O$_2$–mediated GJIC inhibition. This study suggests that OPE, a material often discarded, may be of value for the future development of functional food products.

Keywords: connexin (Cx) 43, gap-junctional intercellular communication (GJIC), onion peel extract (OPE), quercetin, reactive oxygen species (ROS)

Practical Application: This study demonstrates that onion peel extract (OPE) exhibits a protective effect against the inhibition of gap–junctional intercellular communication (GJIC) mediated by H$_2$O$_2$, which is likely to occur via its antioxidant activity. OPE contains significant concentrations of bioactive phenolic compounds. Reductions in oxidative stress can lead to recovery of GJIC, which has been reported to be implicated in the prevention and treatment of cancers. These findings suggest that onion peel, a common waste product, could be used as potential resources for functional food development. Onion peel could be processed into a quercetin–rich powder or a pill for the prevention of cancer and other oxidative stress-related diseases.

Introduction

Gap–junctional intercellular communication (GJIC) involves the exchange of ions and other cytoplasmic molecules between neighboring cells and contributes to the maintenance of homeostatic balance via the regulation of cellular proliferation, differentiation, and growth (Trosko and Ruch 1998). In normal conditions, most cells of the human body have active and functional GJIC (Kumar and Gilula 1996), whereas carcinogenesis, particularly in its formative stages, is strongly associated with GJIC

inhibition (Trosko and Ruch 1998). Studies have shown that GJIC inhibition can be reversed by foods containing high concentrations of polyphenols. Apple extracts and vitamin C have been shown to have protective effects against hydrogen peroxide (H$_2$O$_2$)-mediated GJIC inhibition (Lee and others 2004). In addition, kiwifruit cultivars and their active phenolic compounds have been found to reverse H$_2$O$_2$-mediated GJIC inhibition in WB-F344 rat liver epithelial (RLE) cells (Lee and others 2010).

The presence of excessive levels of reactive oxygen species (ROS) can cause oxidative damage and result in the dysregulation of GJIC. Activation of MAPK/ERK kinase (MEK) and extracellular signal-regulated protein kinase 1/2 (ERK1/2) by H$_2$O$_2$ can induce hyper-phosphorylation of connexin (Cx)43, a major component of transmembrane protein complexes (Lee and others 2004; Lee and others 2010), leading to the inhibition of GJIC. The phosphorylation of Cx43 has been reported to induce conformational changes in membrane-bound proteins that inhibit GJIC (Bruzzone and others 1996; Na and others 2000; Kang and others 2001). While H$_2$O$_2$ is known to inhibit GJIC in WB-F344 RLE cells, this inhibition can be reversed by various antioxidants, such as catalase or natural phytochemicals (Kang and others 2008).

The onion is the second most important crop worldwide (Benitez and others 2011). It has been reported that onion peels

are wasted during processing (Benitez and others 2011; Gawlik-Dziki and others 2013). Benitez and others reported that about 450000 tons of onion waste is produced in Europe (Benitez and others 2011). Since onion wastes are not suitable for other uses, such as fodder or organic fertilizer, these wastes generate huge environmental problems (Benitez and others 2011). In this regard, this study was designed to provide scientific evidence on the application of onion wastes. To this end, we compared health beneficial effects of the onion peel extract (OPE), which was generally discarded, with the onion flesh extract (OFE).

Naturally occurring antioxidants have been shown to exhibit protective effects against carcinogenesis, underlining the role of ROS-mediated oxidative damage during the development of cancers. Onion has been reported to confer health benefits via its antioxidative, anticarcinogenic, and antiinflammatory activities (Slimestad and others 2007). These effects are likely attributable to the presence of polyphenolic compounds such as flavonoids. Recently, a number of clinical and animal studies have demonstrated that onion peel, which contains high concentrations of quercetin, exhibits effects that are beneficial for human health (Jung and others 2011; Lee and others 2011; Lee and others 2012). Indeed, onion peel supplementation has been shown to improve blood

lipid profiles, glucose levels, and blood pressure in male smokers (Lee and others 2011). Supplementation with OPE also regulates the expression of genes associated with cholesterol metabolism, leading to reductions in LDL cholesterol and an increase in HDL cholesterol in rats fed on a high-fat diet (Lee and others 2012). In addition, OPE improves hyperglycemia and insulin resistance by enhancing the expression of the muscular insulin receptor and GLUT4 in high fat diet/streptozotocin-induced diabetic rats (Jung and others 2011).

Flavonoids in OPE could also be acting as direct kinase inhibitors. Recently, a number of studies have reported that various flavonoids in food can act as direct kinase inhibitors (Lee and others 2008; Kim and others 2011; Choi and others 2013). Quercetin, the most abundant flavonoid in OPE, has been reported to directly inhibit Raf and MEK1 (Lee and others 2008), and other flavonoids in OPE, such as rutin and isorhamnetin, have also been reported to inhibit EGFR and MEK1, respectively (Kim and others 2011; Choi and others 2013). These results suggest the possibility that various flavonoids in OPE might be acting as direct kinase inhibitors upstream of the ERK1/2 and Cx43-mediated signaling pathway as well as antioxidants, thereby improving oxidative stress-mediated GJIC inhibition during tumor promotion. Accordingly, we aimed to investigate the effect of OPE and OFE on oxidative stress-mediated GJIC inhibition and the mechanism responsible, using the WB-F344 RLE cell line. In addition, we aimed to identify the major components of OPE or OFE responsible for the protective effect against GJIC.

Materials and Methods

Materials

Acetonitrile, methanol, and trifluoroacetic acid were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Dimethyl sulfoxide (DMSO), quercetin, rutin, isorhamnetin, quercitrin,
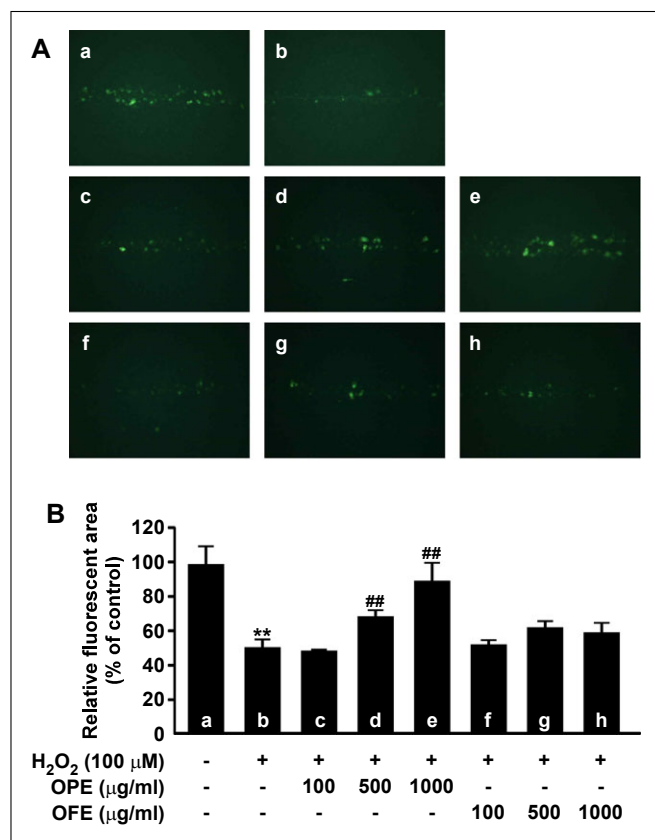

Figure 1–OPE exerts a stronger restorative effect than OFE on H_2O_2-mediated GJIC inhibition in WB-F344 RLE cells. GJIC was assessed using the SL/DT technique, and imaged using an inverted fluorescence microscope (100×). Cells were stained with lucifer yellow. (A) Image of GJIC following treatments. (B) Fluorescent area was measured, and the fluorescent area in the untreated control was normalized to 100%. The relative fluorescent area of each group was calculated as a percentage relative to the controls. Data are expressed as means ± SD values from 3 independent experiments. The asterisks (**) indicate a significant difference between the control group and the group treated with H_2O_2 alone ($P < 0.01$), and the sharps (##) indicate a significant difference between groups treated with H_2O_2 alone and those treated with H_2O_2 plus OPE or OFE ($P < 0.01$).

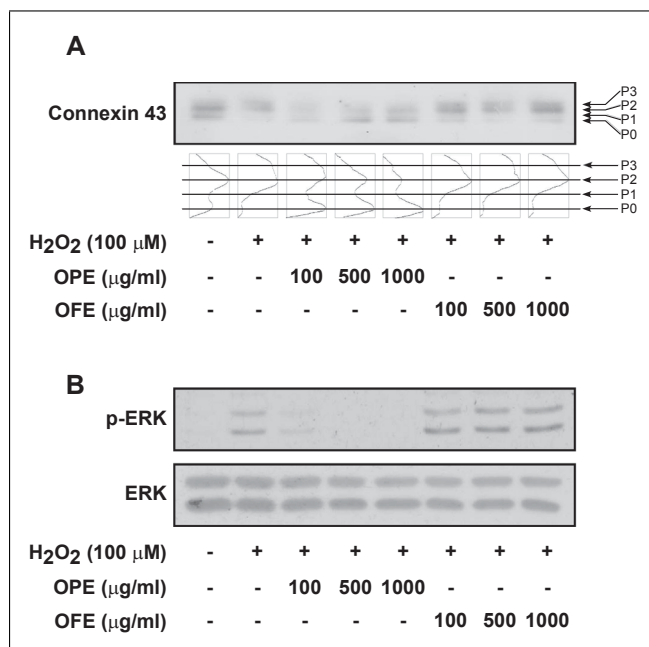Figure 2–OPE, but not OFE, markedly suppresses H_2O_2-induced phosphorylation of Cx43 and ERK1/2 in WB-F344 RLE cells. (A) The phosphorylation status of Cx43 was evaluated by Western blot analysis. The diagrams show the intensity of Cx43 bands from P0 to P3, which were quantified by using Image J software. (B) The levels of phosphorylated ERK1/2 and total ERK1/2 were evaluated by Western blot analysis.

kaempferol, 1,1-diphenyl-2-picrylhydrazyl, and lucifer yellow were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). 2',7'-Dichlorofluorescein-diacetate was obtained from Invitrogen (Carlsbad, Calif., U.S.A.). The antibodies against phosphorylated ERK1/2 (Tyr202/Tyr204) and total ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., U.S.A.), and the antibody against connexin 43 was purchased from Zymed (San Francisco, Calif., U.S.A.).

Preparation of onion peel and onion flesh extracts

Onions (*Allium cepa L.* grown in Jeollanam-do province, Republic of Korea), which were harvested in June 2013, were purchased from a national agricultural and fishery market (Anyang, Republic of Korea). The onions were immediately divided into flesh and peel, which were then freeze-dried (Ilsin, PVTFD 20R, Republic of Korea). Freeze-dried flesh and peel were extracted with 70% (v/v) methanol at room temperature for 24 h, and filtered through filter paper (Whatman Nr. 1). The filtrate was then collected and evaporated (EYELA, NVC-2100, Japan) at 40 °C to remove methanol. The residue was freeze-dried (Ilsin, PVTFD 20R, Republic of Korea) before being powdered with a mortar and pestle, and stored at −20 °C.

Cell culture

The WB-F344 RLE cell line was kindly provided by Dr. J.E. Trosko (Michigan State Univ., East Lansing, Mich., U.S.A.). Cells were cultured in Eagle's modified essential medium (EMEM; Gibco, Gaithersberg, Md., U.S.A.), supplemented with 10% FBS (Gibco, Gaithersberg, Md., U.S.A.) at 37 °C in a 5% CO_2 humidified incubator (Forma Scientific, Marietta, Ohio, U.S.A.).

GJIC bioassay

GJIC was measured using a scrape-loading/dye-transfer (SL/DT) technique as previously described (el-Fouly and others 1987). Briefly, WB-F344 RLE cells were seeded in 3.5-cm culture dishes (BD Bioscience, Heidelberg, Germany) and incubated until confluence. Cells at confluence were incubated with 100 μM of H_2O_2, supplemented with the onion extracts (OPE or OFE) or individual compounds (quercetin, rutin, and isorhamnetin) for 1 h. Cells were then washed twice with prewarmed phosphate buffer solution (PBS) (2 mL), and Lucifer yellow (1 mg/mL) was added. Cells were then scraped using a scalpel with a surgical-steel blade. After incubation with lucifer yellow solution for 3 min, the cells were washed 3 times with prewarmed PBS (2 mL) and fixed with a 4% formaldehyde solution (2 mL). Fluorescence was observed through an inverted fluorescence microscope at 200× magnification (Olympus, Okaya, Japan), and fluorescence was quantified using Image J software (NIH, Bethesda, Md., U.S.A.).

Western blot analysis

Western blot analysis was performed to assess the phosphorylation of Cx43 and ERK1/2. Cells were seeded and cultured in EMEM supplemented with 10% FBS until 90% confluence. After starvation for 24 h with serum-free EMEM, the cells were treated with onion extracts (OPE or OFE) or individual phenolic compounds (quercetin, rutin, and isorhamnetin) for 30 min, followed

	IC 50 (μg/ml)
OPE	148.0
OFE	-
Vitamin C	45.7

Figure 3–The free radical-scavenging activity of OPE is greater than that of OFE. (A) DPPH radical scavenging activity was evaluated. Absorbance at 515 nm was measured and the absorbance in the nonsample treated control (0 μg/mL) was normalized to 100%. The relative rate of absorbance was calculated as percentage relative to the control. (B) DCF-DA assay was conducted to confirm antioxidant capacity of OPE, OFE, and vitamin C in WB-F344 RLE cells. Fluorescence intensity was measured and the relative rate of fluorescence at 2 h was expressed as means ± SD values from 3 independent experiments. The asterisks (**) indicate a significant difference between the control group and the group treated with H_2O_2 alone ($P < 0.01$), and the sharps (##) indicate a significant difference between groups treated with H_2O_2 alone and those treated with H_2O_2 plus OPE, OFE, or ascorbic acid ($P < 0.01$).

Table 1–Total phenols, total flavonoids, and major polyphenols concentrations in OPE and OFE.

Compounds	OPE	OFE
Total phenols (GAE mg/100 g)[a]	25657 ± 310.3	587 ± 12.0
Total flavonoids (CE mg/100 g)[b]	13214 ± 341.6	72 ± 2.6
Major polyphenols (mg/100 g)[c]	5910 ± 27.7	8.1 ± 0.1
Quercetin (mg/100 g)	4058 ± 33.4	7.7 ± 0.1
Rutin (mg/100 g)	841.5 ± 7.5	ND
Isorhamnetin (mg/100 g)	818.2 ± 3.7	0.4 ± 0.0
Quercitrin (mg/100 g)	103.6 ± 2.1	ND
Kaempferol (mg/100 g)	88.4 ± 0.4	ND

[a]mg of gallic acid equivalents (GAE) per 100 g fresh weight as standard.
[b]mg per catechin equivalents (CE) per 100 g fresh weight as standard.
[c]Sum of quercetin, rutin, isorhamnetin, quercitrin, kaempferol.

by a treatment with 100 μM of H_2O_2 for 30 min. The cells were lysed with 1× sample buffer (2% SDS, 5% glycerin, 62.5 mM 2-amino-2-(hydroxymethyl) propane-1,3-diol hydrochloride [pH 7.5], 0.05% bromophenol blue, and 10 mM dithiothreitol). Cell lysates were heated at 95 °C for 5 min and centrifuged (14000 rpm, 10 min, 4 °C) to isolate the pellet. Lysates were then separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide gel. Separated proteins were transferred to a 0.45 μm polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Piscataway, N.J., U.S.A.). The membranes were blocked with 5% skim milk for 1 h and incubated with the indicated primary antibodies followed by horseradish peroxidase
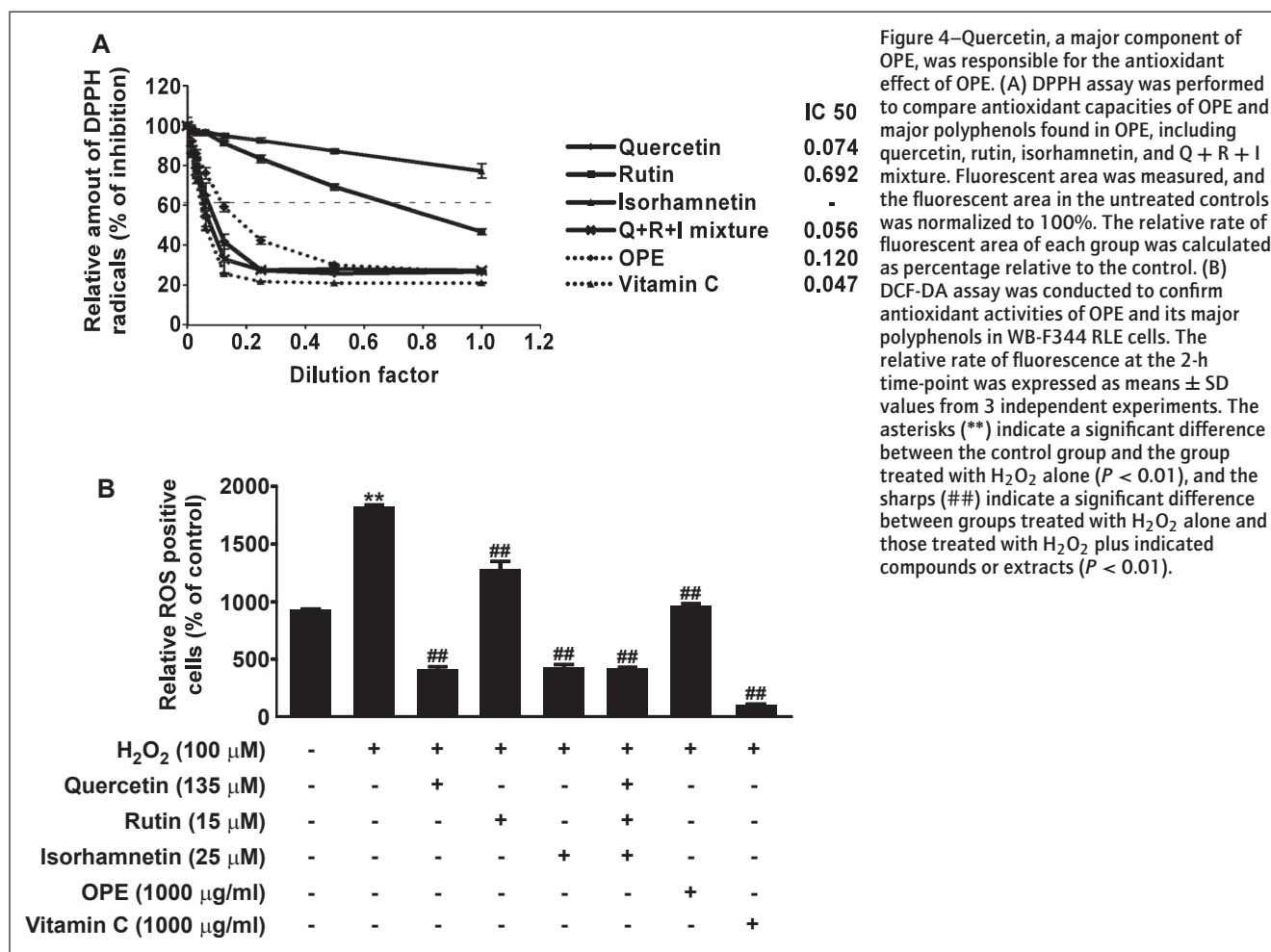
(HRP)-conjugated secondary antibodies. Proteins were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, N.J., U.S.A.).

DPPH assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity was measured as previously described (Lee and others 2010). DPPH was dissolved in 80% methanol to make 0.1 mM solution. DPPH solution (1993.3 μL) was mixed with OPE or OFE (6.7 μL) with various concentrations (0, 10, 25, 50, 100, 250, 500, and 1000 μg/mL), and loaded into 96-well plates. The mixture was vigorously shaken for 30 min in the dark. The DPPH radical scavenging rate was estimated by measuring absorbance at 515 nm using a spectrophotometer (Hitachi, Tokyo, Japan).

DCF-DA assay

WB-F344 RLE cells were cultured until 90% confluence before trypsinization and centrifugation (2000 rpm, 3 min). Supernatant was removed and the cell pellets were resuspended with 20 μM of 2′,7′-Dichlorofluorescein-diacetate (DCF-DA) in prewarmed PBS. Cells were then treated with the indicated extracts or compounds, followed by the addition of 100 μM of H_2O_2. Cleavage of DCF-DA was monitored at indicated time points by measuring fluorescence with excitation at 485 nm and emission at 538 nm using a spectrophotometer (Hitachi, Tokyo, Japan).

Figure 4–Quercetin, a major component of OPE, was responsible for the antioxidant effect of OPE. (A) DPPH assay was performed to compare antioxidant capacities of OPE and major polyphenols found in OPE, including quercetin, rutin, isorhamnetin, and Q + R + I mixture. Fluorescent area was measured, and the fluorescent area in the untreated controls was normalized to 100%. The relative rate of fluorescent area of each group was calculated as percentage relative to the control. (B) DCF-DA assay was conducted to confirm antioxidant activities of OPE and its major polyphenols in WB-F344 RLE cells. The relative rate of fluorescence at the 2-h time-point was expressed as means ± SD values from 3 independent experiments. The asterisks (**) indicate a significant difference between the control group and the group treated with H_2O_2 alone ($P < 0.01$), and the sharps (##) indicate a significant difference between groups treated with H_2O_2 alone and those treated with H_2O_2 plus indicated compounds or extracts ($P < 0.01$).

Quantification of total phenolic and flavonoid contents

The total phenolic content of OPE and OFE were determined using a modified Folin-Ciocalteu method (Singleton and others 1998). Samples (OPE and OFE) or a standard solution was diluted and transferred into a 15-mL tube containing 2.6 mL of deionized water. A reagent blank was also prepared using water. Folin–Ciocalteu phenol reagent (0.2 mL) was added into the mixture. After shaking for 6 min, 7% Na_2CO_3 solution (2 mL) was added, and immediately mixed with deionized water. Following incubation for 90 min at 23 °C, the absorbance relative to that of the prepared blank was read at 750 nm using a spectrophotometer (Model 8453, Agilent Technologies, Palo Alto, Calif., U.S.A.). The total phenolic contents were expressed in mg of gallic acid equivalents (GAE) per 100 g extract weight as a standard curve.

The total flavonoid contents of OPE and OFE were measured using a colorimetric method (Meyers and others 2003). Samples (OPE and OFE) or a standard solution (1 mL) was added to a 15-mL tube containing 4 mL of deionized water. Then 0.3 mL of 5% $NaNO_2$ was added to the mixture. After incubation for 5 min at 23 °C, 0.3 mL of 10% $AlCl_3\cdot6H_2O$ was added. After incubation for 6 min at 23 °C, 2 mL of 1 M NaOH was added, and the total volume was made up to 10 mL with deionized water. The absorbance of the solution compared with a blank at 510 nm was measured immediately using a spectrophotometer (Model 8453, Agilent Technologies, Palo Alto, Calif., U.S.A.). The total flavonoid contents were expressed as mg of catechin equivalents (CE)/100 g of extract weight as a standard curve.

High-performance liquid chromatography (HPLC) analysis

HPLC analysis of individual compounds was performed as previously described (Crozier and others 1997a, 1997b). Briefly, OPE or OFE (0.2 g) was homogeneously dissolved in 10 mL of methanol. The mixtures were filtered through a 0.45 μm filter (Sartorius, Gotettingen, Germany) for HPLC analysis. The mobile phase gradient consisted of water adjusted to pH 2.5 with trifluoroacetic acid (*solution A*) and 100% acetonitrile solution (*Solution*

B). The following gradient was used: 0 to 25 min, 80% to 60% *Solution A*; 25 to 26 min, 60% to 80% *Solution A*; 26 to 30 min, 80% *Solution A*. The operating conditions were as follows: flow rate, 1.0 mL/min; column temperature, 25°C; injection volume, 10 μL; with monitored at 365 nm. HPLC analysis was carried out using 1200 Series HPLC System (Agilent, Palo Alto, Calif., U.S.A.), and analytical separations were performed using an Capcell Pak C18 UG120 column (4.6 mm i.d. × 150 mm, 5 μm, Shiseido, Inc., Tokyo, Japan). The sample concentrations were calculated by comparing the peak areas with external calibration curves.

Statistical analysis

Data are expressed as means ± SD, and Student's *t*-test was used for statistical comparisons. A probability value of $P < 0.05$ was used as the criterion for significance.

Results and Discussion

OPE restores H_2O_2-mediated GJIC inhibition to a greater extent than OFE

GJIC is essential for the maintenance of tissue homeostasis between adjacent cells by exchanging ions, signaling molecules, nucleotides, and other small molecules (Trosko and Ruch 1998). It therefore plays a critical role in cell proliferation, differentiation, and growth of cancer cells (el-Fouly and others 1987). We evaluated the effects of OPE and OFE on H_2O_2-mediated GJIC inhibition using an SL/DT technique with WB-F344 RLE cells. Treatment with H_2O_2 (100 μM) inhibited GJIC by approximately 50% compared to control cells without H_2O_2 treatment (Figure 1A and B). This finding is consistent with studies conducted by others (Crozier and others 1997a; Singleton and others 1998; Rudolf and others 2007). The inhibition was significantly restored by treatments with OPE at 500 and 1000 μg/mL, while OPE at 1000 μg/mL was sufficient to restore GJIC inhibition to a level similar to that of control cells without H_2O_2 treatment (Figure 1A and B). However, treatment of OFE up to 1000 μg/mL
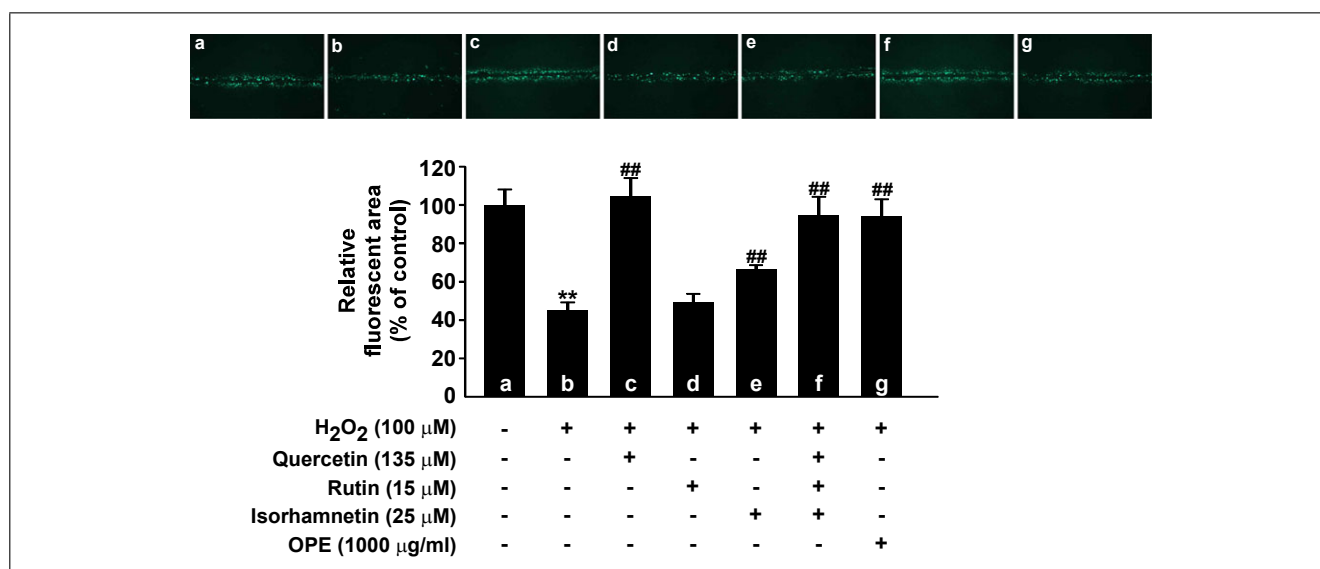

Figure 5–Stronger restorative effect of OPE against H_2O_2-mediated GJIC inhibition was attributed to quercetin in WB-F344 RLE cells. GJIC was assessed using the SL/DT technique, and imaged using an inverted fluorescence microscope (100×). Cells were stained with lucifer yellow. Image of GJIC following treatments, and fluorescent area was measured, and the fluorescent area in untreated controls was normalized to 100%. The relative rate of fluorescent area of each group was calculated as percentage relative to the control. Data are expressed as means ± SD values from 3 independent experiments. The asterisks (**) indicate a significant difference between the control group and the group treated with H_2O_2 alone ($P < 0.01$), and the sharps (##) indicate a significant difference between groups treated with H_2O_2 alone and those treated with H_2O_2 plus OPE or OFE ($P < 0.01$).

did not affect GJIC inhibition induced by H_2O_2 (Figure 1A and B). OPE therefore exhibits a stronger recovery effect against GJIC inhibition induced by oxidative stress than OFE.

OPE suppresses H_2O_2-dependent phosphorylation of Cx43 and ERK1/2

H_2O_2 has been reported to induce phosphorylation of Cx43 and ERK1/2, which has been implicated in the disruption of GJIC (Upham and others 1997; Kang and others 2000). Accordingly, we aimed to investigate whether OPE-mediated GJIC restoration that was normally increased by oxidative stress was associated with decreased Cx43 and ERK1/2 phosphorylation. Antibodies specific for Cx43 were specific for 4 different bands according to phosphorylation status, P0, P1, P2, and P3 (Lee and others 2010). While the most motile band (P0) is indicative of nonphosphorylated Cx43, the least-motile band (P3) represents the most hyper-phosphorylated Cx43. We found that treatment of H_2O_2 (100 μM) causes hyper-phosphorylation of Cx43, evidenced by greater expression of the P3 band compared to P2, P1, and P0 (Figure 2A). Interestingly, treatment with OPE (100, 500, and 1000 μg/mL) dose-dependently shifted the pattern of the bands by enhancing the density of P0 and reducing the density of P3. This suggests that OPE suppresses H_2O_2-induced hyper-phosphorylation of Cx43 (Figure 2A). However, the same amounts of OFE (100, 500, and 1000 μg/mL) did not affect H_2O_2-induced hyper-phosphorylation of Cx43, as seen as a higher density of P3 band (Figure 2A).

Consistent with the finding that OPE only regulates H_2O_2-induced Cx43 phosphorylation, OPE (at 100, 500, and 1000 μg/mL), but not OFE, inhibited ERK1/2 phosphorylation induced by 100 μM of H_2O_2 (Figure 2B). Collectively, these results suggest that OPE, but not OFE, suppresses H_2O_2-induced Cx43 hyper-phosphorylation by inhibiting ERK1/2 phosphorylation, which likely contributes to reverse oxidative stress-mediated GJIC inhibition.

OPE exhibits stronger radical scavenging activity than OFE

We next examined whether the recovery of ROS-mediated GJIC inhibition by OPE was due to its ROS scavenging activity. We measured and compared the radical scavenging activities of OPE and OFE using DPPH and DCF-DA assays. The DPPH radical scavenging activity of OPE and OFE were evaluated and compared with the natural antioxidant vitamin C. We found that OPE (0, 10, 25, 50, 100, 250, 500, and 1000 μg/mL) dose-dependently attenuated DPPH radical formation (Figure 3A). The half maximal inhibitory concentration (IC_{50}) value of OPE was 148 μg/mL, which was higher than that of vitamin C at 45.7 μg/mL. The radical scavenging activity of OPE was not as potent as vitamin C, but stronger than that of OFE. In fact, treatment with OFE at 0, 10, 25, 50, 100, 250, 500, and 1000 μg/mL doses was unaffected by DPPH radical regulation (Figure 3A).

To confirm the intracellular radical scavenging activity of OPE and OFE, a DCF-DA assay was performed using WB-F344 RLE cells. The cells were treated with OPE, OFE, and vitamin C at 1000 μg/mL for 2 h in the presence of H_2O_2 (100 μM). H_2O_2-mediated intracellular ROS formation was approximately 2 times higher than in control cells without H_2O_2 treatment (Figure 3B). Consistent with the finding from the DPPH assay, the intracellular ROS scavenging activity of OPE was greater than OFE. OPE mitigated the formation of ROS by 50% compared to the control cells without H_2O_2 treatment. OFE also attenuated H_2O_2-mediated ROS formation by 10% compared to H_2O_2-treated control cells (Figure 3B). These results suggest that OPE exhibits a stronger ROS scavenging ability than OFE, and the strong antioxidant capacity of OPE is likely to contribute to the suppression of ERK1/2 phosphorylation and subsequent hyper-phosphorylation of Cx43, thereby protecting against oxidative stress-mediated GJIC inhibition.

OPE contains higher levels of polyphenols and flavonoids than OFE

OPE exhibited a greater antioxidant capacity than OFE, as evident through intracellular ROS scavenging analysis, and subsequently decreased Cx43 phosphorylation and GJIC restoration in H_2O_2-treated WB-F344 RLE cells. We further attempted to evaluate whether GJIC restoration by OPE was attributable to the presence of active polyphenols and flavonoids. We analyzed the total phenolic contents of OPE and OFE using the Folin-Ciocalteu method (Arnous and others 2001). We found that the total levels of polyphenols in OPE was approximately 44 times higher than that in OFE (Table 1). Consistent with the higher levels of total phenolic contents, OPE was found to contain approximately 183 times more total flavonoids than OFE (Table 1). HPLC was then performed to identify the major polyphenols present in OPE and OFE. Quercetin, rutin, isorhamnetin, quercitrin, and kaempferol were identified as being the most abundant in both OPE and OFE. Interestingly, the sum of the total quantity of the 5 polyphenols was 738 times higher in OPE than OFE per unit weight (Table 1). Among these polyphenols, quercetin was found to be the most abundant compound in both OPE and OFE, but the absolute amount of quercetin in OPE was 579 times higher than in OFE. While OFE shows very low levels of other flavonoids, OPE has greater amounts of rutin, isorhamnetin, quercitrin, and kaempferol. However, these flavonoids exist in relatively minor portions compared to quercetin. In summary, quercetin is the most abundant polyphenol found in OPE, which is likely responsible for H_2O_2-induced GJIC inhibition via suppression of Cx43 and ERK1/2 phosphorylation in WB344 RLE cells.

The antioxidant capacity of OPE is attributable to the major polyphenol quercetin

To determine whether the antioxidant capacity of OPE could be attributable to its polyphenol content, we compared the DPPH radical scavenging activity of major OPE components (quercetin, rutin, and isorhamnetin) and a mixture of the 3 compounds to mimic the constituents of OPE. We observed that 1000 μg/mL of OPE contains 135 μM quercetin, 15 μM rutin, and 25 μM isorhamnetin. Therefore, the same concentrations of each polyphenolic component and a mixture of quercetin 135 μM (Q), rutin 15 μM (R), and isorhamnetin 25 μM (I) (referred to as Q + R+ I mixture) were used to examine the antioxidant capacity. The DPPH radical scavenging activity was similar between quercetin and the Q + R + I mixture, showing IC_{50} values of 0.074 and 0.056 μg/mL, respectively. These values were similar to that of vitamin C (0.047 μg/mL), and even lower than that of OPE (0.120 μg/mL) (Figure 4A). In addition, quercetin, isorhamnetin, and the Q + R + I mixture significantly reduced H_2O_2-mediated ROS formation in WB-F344 RLE cells, and even reduced it further than that observed during OPE treatment (Figure 4B). These findings suggest that the antioxidant capacity of OPE is likely attributable to its higher levels of quercetin, and moderate levels of rutin and isorhamnetin.

The DPPH radical scavenging activity of quercetin was greater than the same quantity of OPE, while the DCF-DA assay revealed that the intracellular ROS scavenging activity of isorhamnetin as well as quercetin and the Q + R + I mixture were also greater than that of OPE in WB-F344 RLE cells (Figure 4A and B). The Q + R + I mixture exhibited a greater antioxidant capacity by reducing DPPH radical formation and intracellular ROS formation due to the synergistic effects of quercetin and other antioxidative polyphenols. This suggests that quercetin, isorhamnetin, and the Q + R + I mixture eliminate H_2O_2 and other intracellular free radicals. OPE also reduced intracellular ROS to similar levels as the untreated control cells, but the antioxidant capacity was not as strong as quercetin, isorhamnetin, or the Q + R + I mixture. This could be due to of the presence of other minor polyphenols with no antioxidant capacity present in OPE. Many previous studies have reported that an adequate presence of ROS is required for healthy cellular functions, such as maintenance of the cellular innate immune defense system (Conner and others 2002; Leto and Geiszt 2006; Rada and Leto 2008). Therefore, an antioxidant capacity similar to that of control cells without H_2O_2 treatment could be more beneficial for human health than excessive ROS elimination.

Quercetin is responsible for the protective effect of OPE against H_2O_2-induced GJIC inhibition

We further evaluated the effects of OPE, its major polyphenols, and a representative mixture against H_2O_2-mediated GJIC inhibition. OPE, quercetin, and the Q + R + I mixture similarly reversed H_2O_2-mediated GJIC inhibition. Isorhamnetin also significantly restored GJIC inhibition induced by H_2O_2. However, OPE, quercetin, and the Q + R + I mixture restored GJIC inhibition to a greater extent than isorhamnetin (Figure 5). Rutin, one of the major components of OPE, did not reverse GJIC inhibition induced by oxidative stress, which was consistent with an observed weaker antioxidant capacity compared to quercetin and isorhamnetin. Collectively, we found that quercetin is a major factor responsible for the protective effect of OPE against oxidative stress–mediated GJIC inhibition in WB-F344 RLE cells. OPE-mediated GJIC restoration is likely associated with the antioxidant capacity of polyphenols, especially quercetin.

Conclusion

Our results have demonstrated that compared to OFE, OPE exhibits a stronger protective effect against GJIC inhibition by suppressing Cx43 and ERK1/2 phosphorylation during oxidative stress–mediated tumor proliferation, and that this effect is attributable to greater levels of quercetin present in OPE. We conclude that peel extract of onion protects against H_2O_2-mediated GJIC inhibition in WB-F344 RLE cells, suggesting a potential chemopreventive role for oxidative stress–mediated carcinogenesis. This may lead to the development of chemopreventive functional food supplements using onion peel, a waste material that is often discarded during food preparation.

Acknowledgments

Disclosure Statement

The authors declared no conflict of interest.

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