The Protective Effect of Quercetin-3-O-β-D-Glucuronopyranoside on Ethanol-induced Damage in Cultured Feline Esophageal Epithelial Cells

Jung Hyun Cho1,*, Sun Young Park1,*, Ho Sung Lee1, Wan Kyunn Whang2, and Uy Dong Sohn1

Departments of 1Pharmacology, 2Pharmacognosy, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea

Quercetin-3-O-β-D-glucuronopyranoside (QGC) is a flavonoid glucoside extracted from Rumex Aquaticus Herba. We aimed to explore its protective effect against ethanol-induced cell damage and the mechanism involved in the effect in feline esophageal epithelial cells (EEC). Cell viability was tested and 2',7'-dichlorofluorescin diacetate assay was used to detect intracellular H2O2 production. Western blotting analysis was performed to investigate MAPK activation and interleukin 6 (IL-6) expression. Exposure of cells to 10% ethanol time-dependently decreased cell viability. Notably, exposure to ethanol for 30 min decreased cell viability to 43.4%. When cells were incubated with 50 μM QGC for 12 h prior to and during ethanol treatment, cell viability was increased to 65%. QGC also inhibited the H2O2 production and activation of ERK 1/2 induced by ethanol. Pretreatment of cells with the NADPH oxidase inhibitor, diphenylene iodonium, also inhibited the ethanol-induced ERK 1/2 activation. Treatment of cells with ethanol for 30 or 60 min in the absence or presence of QGC exhibited no changes in the IL-6 expression or release compared to control. Taken together, the data indicate that the cytoprotective effect of QGC against ethanol-induced cell damage may involve inhibition of ROS generation and downstream activation of the ERK 1/2 in feline EEC.

Key Words: Flavonoid, Hydrogen peroxide, ERK, Esophageal epithelial cell, Ethanol

INTRODUCTION

Ethanol is a commonly used and abused substance. Notably, 10~13% ethanol is used in beverage such as wines [1]. Alcohol has diverse effects on human health and organ systems. Notably, ethanol intake injures the functional and structural integrity of the intestinal mucosa [2]. Clinical experience suggests that the frequency of chronic esophagitis is increased in patients who abuse alcohol [3]. Both the induction of gastro-esophageal reflux and disordered motility of the esophagus caused by acute ethanol ingestion may promote the development of mucosal lesions [3-5]. Reactive oxygen species (ROS) participate and regulate diverse downstream signaling pathways leading to specific cellular functions [6,7] such as growth, metabolic rate, cell division, necrosis, apoptosis and aging processes [8,9]. Oxygen-derived free radicals have been known to play a key role in the generation of gastrointestinal diseases, including the acid-related peptic diseases and inflammatory disorders [10,11]. It was demonstrated that cell damage caused by free radicals in gastric or esophageal mucosa can be prevented by the administration of free radical scavengers [12-14].

Many cellular responses to ethanol are mediated by the modulation of mitogen activated protein kinases (MAPK) signaling [15,16]. MAPK signaling cascades regulate important cellular processes including gene expression, cell proliferation, cell survival and death, and cell motility [17] and the induction of most cytokine genes requires activation of the ERK and p38 MAPK in response to a variety of extracellular stimuli [18,19]. ERK has been classically associated with growth and differentiation inducing signals, whereas p38 MAPK is involved in inflammatory cytokines and environmental stress inducers [20]. Several studies in human and animal models have shown that prolonged alcohol consumption is associated with elevated serum levels of not only tumor necrosis factor (TNF), but also interleukins such as IL-1, IL-6, and IL-8 [21-25]. Enhanced

**ABBREVIATIONS:** ROS, reactive oxygen species; IL, interleukin; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; P38K, phosphatidylinositol 3-kinase; TNF, tumor necrosis factor; HPLC, high performance liquid chromatography; DMSO, dimethyl sulfoxide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.
production of IL-1β and IL-6 has been documented in esophageal tissue of cats with experimental esophagitis [26,27], and both cytokines contribute to reduce esophageal circular muscle contractility [28].

Flavonoids are a large heterogeneous group of benzo-γ-pyron derivatives which are present in fruits, vegetables and medicinal herbs. Flavonoids have received a great deal of attention over the last several decades, and several biological activities including antioxidant, apoptosis-induction and anti-inflammatory effects have been identified [29,30]. Plant-originated flavonoids are highly gastroprotective against gastric mucosal lesions induced by ethanol in rats in vivo [31]. Among them, quercetin (3,5,7,3′,4′-pentahydroxy flavon) has been known to possess a broad range of pharmacological properties, including anti-inflammatory [32], antioxidative [33] and anti-proliferative activities [34]. It has been reported that quercetin exerts potential gastrointestinal protective effect against ethanol-induced cellular damages [35] and prevents the HCl- as well as ethanol-induced gastric mucosal injuries [36]. Quercetin-3-O-β-D-glucuronopyranoside (QGC) is a flavonoid glucoside extracted from *Rumex aquatica* [37]. In our previous study, QGC was more potent than quercetin on inhibition of experimental reflux esophagitis and indomethacin-induced gastritis in rats [37]. In another previous study, QGC enhanced antioxidant enzyme defense systems via heme oxygenase-1 (HO-1) expression and NF-E2-related factor 2 (Nrf2) translocation involving both ERK and PI3K-Akt pathways as well as PKC pathways in esophageal epithelial cell (ECC) [38]. In this study, we aimed to investigate the mechanism of the cytoprotective effect of QGC against cell damage induced by 10% ethanol in cultured feline ECCs. As cell damage factors induced by 10% ethanol, we investigated intracellular ROS production, MAPK activation and IL-6 production and expression in this study.

**METHODS**

**Materials**

QGC (molecular weight: 477), which isolated from the herba of *Rumex aquatica* and its purity was 96~97%, was provided by Pharmaco Botany Resources Laboratory (Dr. Whang, Chung-Ang Univ., Seoul, Korea) and dissolved in serum free DMEM containing 0.01% dimethyldioxol (DMSO). Fetal bovine serum (FBS), antibiotic-antimycotic (penicillin, streptomycin, amphotericin B), trypsin-EDTA, Dulbecco’s modified Eagle’s medium (DMEM), 0.1 N hydrochloric acid and phosphate-buffered saline (PBS) were from Sigma Chemical Co. (St. Louis, MO, USA); human IL-6 enzyme immunometric assay kit was from assay designs (Ann arbor, MI, USA).

**Extraction and isolation of QGC from *Rumex aquatica* herb**

Fresh folium (600 g) of *Rumex aquatica* (Korean name: Todaehwang) was extracted with ethanol under sonicator. After filtration, the ethanol solution was evaporated under a vacuum to yield an ethanol extract (72 g). The extract was partitioned between chloroform and water to give a chloriform-soluble fraction (16 g) and a water-soluble fraction (54 g). Based on target-guided fractionation, the water-soluble fraction was chromatographed on Sephadex LH 20 by elution with 50% methanol to give sub-fraction 1, 2, 3, 4. A portion of sub-fraction 2 was chromatographed with an ODS column using 30% methanol as eluent to give QGC (1.2 g) at a purity of 96~97% by HPLC.

QGC is yellow amorphous powder; IR (KBr) cm⁻¹: 3385 (OH), 1657 (C=O), 1502 (aromatic ring), 1052 (C−O); FAB-MS (neg.) m/z: 477[M-H]⁻, 301 [M-Glu]⁻, 300 [M-H]⁻; ¹H-NMR and ¹³C-NMR data were consistent with literature values [39].

**Preparation of cat esophageal epithelial tissue squares**

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chung-Ang University, in accordance with the guide for the Care and Use of Laboratory Animals in Seoul, Republic of Korea. Adult cats of either sex weighing between 2.5 and 4.0 kg were anesthetized with Zoletil 50 (12.5 mg/0.25 ml/kg), which composed of tiletamine and zolazepam, and euthanized with an overdose of 25% urethane (Aldrich, St. Louis, MO, USA). Then, the abdomen was opened with a midline incision and the esophagus was excised, cleaned and maintained in Krebs buffer with the following composition: 118 mM NaCl, 24 mM NaHCO₃, 1.2 mM KH₂PO₄, 4.8 mM KCl, 2.5 mM CaCl₂, 11 mM glucose and 1.2 mM MgSO₄. The esophagus was opened along the lesser curvature. The location of the squamocolumnar junction was identified and the mucosa was peeled off. The submucosal connective tissue was then removed by micro spring scissors. The mucosa from the esophagus was sliced off into 0.5 mm thick sections with a Stadie Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA, USA). The last slices were cut into 2×2 mm tissue squares with scissors.

**Primary culture and identification of EECs**

EECs were successfully isolated from mucosa and subcultured as previously described [40]. Briefly, the sliced tissue was then placed into DMEM supplemented with 10% FBS containing 100 U/ml penicillin, 0.1 mg/ml streptomycin,
cin and 0.25 μg/ml amphotericin B, then incubated in a humi-
dified atmosphere of 5% CO₂ and 95% air at 37°C. After 10 days, the medium was exchanged with fresh DMEM con-
taining 10% FBS. After reaching confluence, cells were de-
tached with 1% Trypsin-EDTA in HBSS with bicarbonate. Cells were then counted, seeded at 2×10⁴ cells/ml on
100-mm culture dishes and maintained in DMEM containing
10% FBS. The medium was changed every 48 h until the cells reached confluence. Experiments were performed on cells at passage 3 or 4.

In order to confirm typical epithelial morphology of the primary-cultured EECs, cells were captured and confirmed using a phase contrast microscope (model ULWCD 0.30 Olympus, Tokyo, Japan) and a digital closed-circuit video camera (CCD color camera, Toshiba, Tokyo, Japan) con-
ected to a Macintosh computer (Apple, Cupertino, CA) with NIH Image 1.57 software (National Institutes of Health, Bethesda, MD). In order to characterize epithelial cells and to exclude contamination by smooth muscle cells and fibroblasts, cells were fixed with 10% formalin containing
0.1% Triton X-100 at passage 1 and 2, and identified by an indirect immunofluorescent staining method using a cytokeratin monoclonal antibody from Dako and captured and confirmed by a Olympus BX51 microscope.

**Measurement of cell viability**

Cell viability was determined by the conventional MTT reduction assay as previously described [40]. In this assay, viable cells convert MTT to insoluble blue formazan crystals
by the mitochondrial respiratory chain enzyme succinate dehydrogenase. Cells were seeded at a density of 2×10⁴/
6-well plates and maintained in DMEM containing 10% FBS. Then, cells were made quiescent at confluence by in-
cubation in serum-free DMEM for 24 h, followed by treat-
ment with indicated concentrations of each compound for
the desired time. After the incubation, cells were washed
with PBS three times and treated with MTT solution (final concentration, 5 mg/ml) for 4 h at 37°C. Then, the super-
natant was removed and the formazan crystals were dis-
solved with 500 μl DMSO. Absorbance at 570 nm was
measured with a microplate reader (Molecular Devices, Sunnyvale, CA).

**Measurement of ROS production**

2',7'-Dichlorofluorescein diacetate (DCF-DA) was used to measure the level of intracellular H₂O₂ production induced by ethanol in EECs [40]. Cells were seeded and grown on
6-well plates for 2 day and then serum-starved in DMEM for
24 h. The cells were treated with 50 μM QGC for 3 h and then incubated with H₂O₂-sensitive fluorophore DCF-
DA (20 μM) for 1 h at 37°C in the dark. The cells were then washed with Krebs solution and exposed with ethanol for indicated times at 37°C in the dark. The cells were har-
vested and centrifuged for 5 min at 10,000 rpm at 4°C to
remove the supernatants. After the pellets were resus-
pended with Krebs buffer, DCF fluorescence was measured
using a fluorospectrophotometer using excitation and emis-
sion wavelengths of 485 and 535 nm, respectively (Tecan, GENios Pro).

**Measurement of IL-6 production**

Cells were cultured in 24-well plates until confluent and
then treated with 50 μM QGC for 12 h before incubation with 10% ethanol for indicated times. After the incubation, supernatants were collected at various time points, and cyto-
kine content was measured by an IL-6 enzyme immuno-
metric assay kit. Assays were performed according to the
manufacturer’s instruction.

**Preparation of cell extracts and protein determination**

When the cells reached confluence, they were serum starved by incubation in serum-free DMEM for 24 h. The cells were then stimulated with each compound for in-
dicated time periods or at indicated concentrations. After incubation, the cells were rapidly washed twice with ice-
cold PBS and lysed with ice-cold lysis buffer for 5 min con-
taining: 20 mM Tris- HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1% (w/v) Triton X-100, 0.01% (w/v) SDS, 10 μg/ml leupeptin, 10 μg/ml aproatin, 1 mM phenylmethylsulfonyl
fluoride, and 0.7 μg/ml β-mercaptoethanol. The lysates were scraped with a cell scraper and collected in eyppendorf tubes. They were then sonicated and centrifuged for 10 min at
13,000 rpm at 4°C to remove cellular debris and the super-
natants were collected.

Equal amounts of the protein from each sample were re-
solved on a SDS-polyacrylamide gel by electrophoresis; the
protein concentrations of supernatants were determined with Bradford reagent according to the instructions of the
manufacturer (Bio-Rad Chemical Division, Richmond, CA). Absorption was monitored at 595 nm.

**Western blotting analysis**

Equal amounts of protein from each sample were sub-
jected to electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose (NC) membrane, using a Power Pac 1000 power supply (Bio-Rad, Melville, NY).
To block nonspecific binding, the NC membrane was in-
cubated in 5% nonfat dry milk in PBS for 60 min followed
by three rinses in milk-free PBS. The membranes were in-
cubated for 1 h with shaking with primary antibodies
raised against each phospho-specific ERK 1/2, phospho-
-JNKs, phospho-p38 MAPK and IL-6 protein followed
by three washes with PBS containing 0.05% Tween 20. This was followed by 60 min incubation in horseradish perox-
idase-conjugated secondary antibody. Detection was per-
formed with an enhanced chemiluminescence agent. Mole-
cular masses were estimated by comparison with a pre-
stained molecular mass marker. To confirm uniformity of
protein loading, the same blots were subsequently stripped with western blot stripping buffer and reprobed with ERK
1/2, p38 MAPK, JNK, and GAPDH antibody. Developed
films were scanned and analyzed densitometrically using
Scion Image. Percent of MAPKs activation and IL-6 ex-
pression were calculated as the ratio of phosphorylated
MAPK to total MAPK and IL-6 to actin, respectively.

**Statistical analysis**

Data are expressed as mean±SEM of n separate experi-
ments and differences between means were analyzed by a
Student’s t test (two-tailed), with p<0.05 considered as
significant.
RESULTS

Time-course analysis for the effect of 10% ethanol on cell viability

To assess whether 10% ethanol causes cell death in cultured EECs, serum starved cells were exposed to 10% ethanol for the indicated times and then cell viability was measured using MTT assay (Fig. 1A). There was no significant decrease in cell viability of cultured EECs incubated in ethanol for 10 min compared to control. After exposure to ethanol for 10 min, however, cell viability was decreased to about 70%. In addition, exposure of cells to ethanol for 30 min led to less than 50% cell viability.

Concentration-related protective effect of QGC against the ethanol-induced cytotoxicity

In a previous study, we have reported the chemical structure of QGC compared to quercetin [38]. QGC alone did not exhibit any significant cytotoxicity on EECs at various concentration of 25~200 μM when incubated for 24 h (data not shown). In this time, to assess the cytoprotective effect of QGC against 10% ethanol-induced cell damage, serum starved cells were pre-incubated with 25~150 μM QGC for 12 h and then exposed to 10% ethanol for 30 min, followed by measurement of cell viability (Fig. 1B). Ethanol treatment alone for 30 min caused a significant decrease to about 45% in cell viability. When cells were pretreated with 50~150 μM QGC for 12 h, the viability of cells exposed to ethanol significantly increased to 68%.

Effect of QGC on intracellular H₂O₂ levels

Serum starved EECs were pre-incubated with the presence of 50 μM QGC for 4 h. Cells were then stimulated with 10% ethanol for 10 min and intracellular ROS production was estimated using DCF-DA (Fig. 2). 10% ethanol alone induced significant increase in intracellular ROS production by 135.3% vs. control. However, when cells were pretreated with 50 μM QGC for 4 h, the ROS levels were significantly decreased below about 45%.

Ethanol-induced MAPK activation

To test whether ethanol induces activation of MAPK, serum starved cells were treated with 10% ethanol at the indicated time periods. Ethanol induced the activation of ERK 1/2, which reached maximally at 10 min (Fig. 3A). Longer stimulation with 10% ethanol, only slightly reduced ERK 1/2 phosphorylation. As shown in Fig. 3B, phosphorylation of p38 MAPK did not cause significant changes after treatment of ethanol. Similarly, JNK was also not changed (Fig. 3C).

Effect of QGC and diphenylene iodonium (DPI) on ethanol-induced ERK 1/2 activation

Next, serum starved EECs were pre-incubated in the presence of 50 μM QGC for 12 h and then stimulated with 10% ethanol for 10 min. Pretreatment with QGC inhibited the expression of 10% ethanol-induced ERK 1/2 phosphorylation. To further clarify whether intracellular ROS production is involved in 10% ethanol-induced ERK 1/2 activation, DPI was utilized. Serum starved cells were pretreated with 10 μM DPI for 30 min and then exposed to ethanol.
QGC Protects Ethanol-induced Cell Damage

Fig. 3. Time course of MAPK phosphorylation induced by ethanol. Serum-starved EECs were incubated with ethanol for indicated time periods. Phosphorylation of ERK 1/2 (A), p38 MAPK (B) and JNK (C) were estimated by western blotting analysis. Data are expressed as means±SEM of three experiments. Student’s t-test; *p < 0.05 vs. control.

Fig. 4. Effects of QGC and DPI on ethanol-induced ERK 1/2 phosphorylation. Serum-starved EECs were incubated with QGC (50 μM, 12 h), DPI (a NADPH oxidase inhibitor, 10 μM, 0.5 h), PD98059 (a MEK inhibitor, 30 μM, 1 h) prior to ethanol treatment for 10 min. Phosphorylation of ERK 1/2 was estimated by western blot analysis. Data are expressed as means±SEM of three experiments. Student’s t-test; *p < 0.05 vs. control, #p < 0.05 vs. cells in 10% ethanol alone.

10% ethanol for 10 min. DPI blocked ethanol-induced activation of ERK 1/2 (Fig. 4). These results indicated that the inhibitory effect of QGC on ERK 1/2 activation by ethanol treatment may be mediated by reducing ethanol-induced intracellular ROS production which originate from NADPH oxidase.

Effect of ethanol in the presence or absence of QGC on IL-6 production and expression

To test whether ethanol induces a cytokine response, serum starved EECs were stimulated with 10% ethanol for 30 and 60 min. Ethanol alone did not induce IL-6 protein expression in the absence or presence of QGC when estimated by western blotting (Fig. 5A). Moreover, ethanol also did not induce IL-6 release in the culture medium in the absence or presence of QGC compared to control (Fig. 5B).

DISCUSSION

Alcohol has many effects on the esophagus and stomach, and changes in these two organs can significantly increase morbidity due to alcohol consumption [3]. Ethanol is related to inflammation of the esophagus and stomach [41]. Both acute and chronic alcohol consumption have severe effects on the structure and function of the entire gastrointestinal tract which results in a vicious cycle [42]. Ethanol intake injures the functional and structural integrity of the intestinal mucosa [2]. Previous study showed that ethanol-induced cell injury was dependent on both the concentration of ethanol applied and the duration of exposure in rat gastric epithelial cells. 6~10% of ethanol was used as a cytotoxic agent [43]. In the present study, we also confirmed...
that 10% ethanol induces cytotoxicity to cat EECs.

It is well known that flavonoids, which are natural product of plants, have antioxidative and the antiinflammatory effects [44]. Quercetin has already been reported that antioxidative and antiinflammatory flavonoid [45,46]. In our previous study, QGC, a flavonoid glycoside extracted from *Rumex Aquaticus*, also acts as a non-stressful and non-cytotoxic antioxidant and antiinflammatory flavonoid in rat model [37]. Flavonoids are known as free radical scavengers and cytoprotective compounds [43], and exhibited protection against H2O2-mediated cytotoxicity. It was known that antioxidants protect cells against ethanol-induced cytotoxicity and apoptosis [47]. In our study, QGC also exhibited a protective effect against 10% ethanol which caused EEC death. We have already confirmed that QGC enhances antioxidant enzyme defense systems via HO-1 expression and Nrf2 translocation involving both the ERK and PI3K-Akt pathways as well as partial involvement of PKC pathways in EEC [38]. Mice lacking functional HO-1 exhibit chronic inflammation and increased mortality after lipopolysaccharide challenge [48,49]. Overexpression of HO-1 in cells resulted in a marked reduction in injury and lipopolysaccharide challenge [48,49]. Overexpression of HO-1 in cells resulted in a marked reduction in injury and lipopolysaccharide challenge [48,49].

Several previous studies have indicated that flavonoids exhibit potent down regulation of ROS generation [58,59]. In the present study, our results suggest that QGC acts as a scavenger of intracellular ROS generation induced by 10% ethanol in EECs.

Ethanol exposure causes the depletion of glutathione (GSH) and the formation of ROS [60,61]. It has been shown that ROS causes deleterious effects by oxidizing important structures in the cells [62] or acting as a second messenger stimulating intracellular signaling pathways including MAPK [63]. Modulation of MAPK signaling pathway by ethanol is distinctive, depending on the cell type, ethanol concentration and duration of exposure [15]. Acute exposure to ethanol results in activation of ERK in astrocytes. However, chronic ethanol treatment causes activation of ERK and p38 MAPK leading to increased synthesis of TNF [64]. Ethanol treatment of stromal osteoblasts increases the ROS associated with induction of NADPH oxidase (NOX) and downstream signaling cascades involving sustained activation of ERK [65]. Also, ROS produced by ethanol in liver Kupffer cells and stellate cells as well as in the lung occur, in part, through activation of NADPH oxidase [66-68]. Concordantly, our data also showed that ethanol induced ERK 1/2 activation via NADPH oxidase-derived ROS production and reduction of the ROS generation by QGC may contribute to inhibition of ethanol-induced ERK 1/2 activation in EECs.

Alcohol consumption is associated with elevated serum levels of interleukins such as IL-1, IL-6, and IL-8 [25,69]. Enhanced production of IL-1β and IL-6 has been documented in esophageal tissue of cats with experimental esophagitis. Mucosa from esophagitis patients has the highest concentrations of IL-1β and IL-6, cytokines known to reduce esophageal muscle contractility [28,70]. However, in our study, 10% ethanol in the absence or presence of QGC did not elicit any changes in the level of IL-6 protein expression or release. Although the ethanol did not induce any change of IL-6 level in our experimental condition, ethanol reduced cell viability (45% compared to control) and induced ERK 1/2 activation via intracellular H2O2 production. Conversely, co-treatment with QGC significantly
reversed the reduction of ethanol-induced cell viability from 45% (ethanol alone) to 68% (ethanol with QGC). Moreover, QGC exhibited inhibitory effect on the ROS-dependent ERK 1/2 activation induced by ethanol. Whether or not QGC-induced inhibitory effect on the ROS-dependent ERK 1/2 activation induced by ethanol be explored via further study.

In conclusion, QGC reduce the 10% ethanol-induced cytotoxicity, and inhibits the production of intracellular ROS and ERK 1/2 activation induced by ethanol. Whether or not QGC-induced inhibitory effect on the ROS-dependent ERK 1/2 activation induced by ethanol is involved in the cytoprotective effect of QGC should be determined by further study.

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