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Quercetin protects gastric epithelial cell from oxidative damage *in vitro* and *in vivo*

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L-012 (8-amino-5-chloro-7-phenylpyrido [3,4-d]pyridazine-1,4-(2H,3H)dione) (PubChem CID: 126741)
MTT (Methyl-thiazol Tetrazolium) (PubChem CID: 64965)
DMSO (dimethyl sulphoxide) (PubChem CID: 679)
H2DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate) (PubChem CID: 77718)
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ABSTRACT

Epithelial injury caused by reactive oxygen species (ROS) including H_2O_2 plays a critical role in the pathogenesis of gastric disorders. Therefore, pharmacological intervention targeting *reactive oxygen species* elimination has highly clinical values in therapy of gastric diseases. Although quercetin has been found to possess gastroprotective activity, whether it has a protective activity againress related injury to gastric epithelial cells remains unknown. The aim of the study is herein to investigate a possible protective effect of quercetin against oxidative stress *in vitro* and *vivo*. Human gastric epithelial GES-1 cells were pretreated with quercetin and then challenged with H_2O_2. In vivo *reactive oxygen species* production in acute gastric mocosa injury was assessed using a chemiluminescent probe L-012 (8-amino-5-chloro-7-phenylpyrido [3,4-d]pyridazine-1,4-(2H,3H)dione) after quercetin was administered to mice. In GES-1 cells, pretreatment of quercetin can significantly diminish H_2O_2-induced cell viability loss; decrease intracellular *reactive oxygen species* and Ca^{2+} influx; restore H_2O_2-induced $\Delta\Psi m$ dissipation. It also upregulates peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) expression under the state of oxidative stress, and the downstream cell apoptosis significantly decreased. In vivo, chemiluminescence imaging shows that quercetin attenuates *reactive oxygen species* production and gastric damages in acute gastric mucosal injury. We first reported the evidence that quercetin can protect gastric epithelial GES-1 cells from oxidative damage and ameliorate *reactive oxygen species* production during acute gastric mucosal injury in mice. This might be ascribed to its inhibition of oxidative stress, regulation of mitochondrial dysfunction, initiation of antioxidant defense and inhibition of apoptosis.

1. Introduction

The impact of gastric diseases on human health, such as chronic gastritis, duodenal and gastric ulceration, adenocarcinoma and gastric MALT lymphoma, remains a big issue deserving worldwide attention, due to the growing consumption of nonsteroidal anti-inflammatory drugs (NSAIDs), prevailing *Helicobacter pylori*, psychological stresses, alcohol consumption and cigarette smoking in modern society (Boland et al., 2005).

In all cases, chronic increased oxidative stress is the major gastric pathologic feature and plays an essential role in the multiple progressions of gastric diseases (Bhattacharyya et al., 2014). Under physiological conditions, gastric epithelium is exposed to higher *reactive oxygen species* level which is derived from physical, chemical or microbiological agents from the gastric lumen, far higher than other tissues or biological fluids (Halliwell et al., 2000; Hiraishi et al., 1994). Intracellular accumulation of *reactive oxygen species* is generated as a consequence of incomplete reduction of oxygen in the normal metabolic processes and also directly produced by a range of oxidase enzymes such as NADPH oxidases (Valko et al., 2007). The imbalance of *reactive oxygen species* generation and the endogenous antioxidant defense system leads to oxidative stress (Valko et al., 2007). Among various *reactive oxygen species*, H_2O_2 is stable, small and uncharged molecule, facilitating it to freely diffuse across cell membranes and acts as a physiological second messenger. However, excessive level of H_2O_2 causes detrimental consequences such as apoptosis, necrosis and other oxidative damage (Veal et al., 2007). Therefore, interventions to ameliorate the processes of oxidative

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stress and its related pathway are applicable to many different gastric indications.

Increased dietary intake and botanicals consumption may reduce the risk for gastric mucosal oxidative injury and accordingly draw much attention (Mahady et al., 2005; Patra et al., 2014; Riceevans et al., 1995). More recently, extracts from apple, strawberry and black rice have been reported to possess extraordinary gastric mucosa protective effect, mainly owing to the abundance in polyphenols and their antioxidant activities (Alvarez-Suarez et al., 2011; Graziani et al., 2005; Kim et al., 2011). Dietary antioxidants, being able to scavenge *reactive oxygen species*, chelate metal ion and break lipid chain peroxidation reactions, play a crucial role in maintaining gastric homeostasis and polyphenolic compounds constitute the major class (Handique and Baruah, 2002). One of the most extensively studied groups of such substances is the flavonoids which are abundant in fruits, vegetables and red wine of plant origin. Of the flavonoids, quercetin is a representative compound. Dietary intake of quercetin and other flavonoids can cause much higher plasma concentrations, while under normal culture conditions, they can also be uptaken intracellular in vitro (Manach et al., 2004). Furthermore, extensive bioactive studies found that quercetin exhibits many beneficial effects on human health, including neuroprotective, anticarcinogenic, anti-inflammatory, antiallergic, and antiviral activities, in particular anti-oxidant and free radical-scavenging activities (Boots et al., 2008). Although quercetin has been found to protect gastric mucosa against ischemia/reperfusion, ethanol, indomethacin, cold restraint stress and *H. pylori* in different animal models (Arsic et al., 2010; Gonzalez-Segovia et al., 2008; Kahraman et al., 2003; Mojzis et al., 2001; Yan et al., 2011), whether quercetin has a protective activity against oxidative stress related injury to gastric epithelial cells remains unknown. Herein, in the present study, we investigate whether quercetin protects *reactive oxygen species* induced damage on a human gastric epithelial cell line, GES-1, challenged with oxidative stress mediated by H_2O_2, and explore the possible mechanisms underlining such action. More importantly, we apply a chemiluminescent probe L-012 to assess the effect of quercetin on *reactive oxygen species* production *in vivo*, trying to offer a direct evidence for its effect against oxidative damage to gastric epithelial cells.

2. Materials and methods

2.1. Cell lines, cell culture, and chemicals

Human gastric epithelial cell, GES-1 cell line, was obtained from the Xiangya School of Medicine, Central South University (Changsha, China). Dulbecco's Modified Eagle's Medium (DMEM/HIGH GLUCOSE) was purchased from HyClone (Peking, China) and fetal bovine serum (FBS) was purchased from HyClone (South American). GES-1 was cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 4 mM L-glutamine, and 4500 mg/l glucose and grown in suspension at 37 °C in a 5% CO_2 humidified incubator. Quercetin was purchased from Sigma.

2.2. Cell viability assay

Cells were seeded in 96-well-plate at a density of 1×10^5 cells/ml in growth medium for approximately 16 h before drug was added. After added quercetin with 2% fetal bovine serum for 24 h incubation, cells were exposed to treatments of 400 μM H_2O_2 or phosphate balanced solution (PBS) for 1 h, then the old medium was discarded and new medium with 2% fetal bovine serum was added to plates. Methyl-thiazol Tetrazolium (MTT) was dissolved in PBS at a concentration of 5 mg/ml, 20 μl of the MTT solution was added to each well of 96-well-plate and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. Finally the MTT-containing medium was removed and 150 μl of dimethyl sulphoxide (DMSO) was added to each well. The absorbance was read at 490 nm using a microplate reader (Tecan Safire, Crailsheim, Germany) and for each treatment eight replicate wells were examined. The A490 was taken as an index of cell viability.

2.3. Measurement of intracellular reactive oxygen species

Intracellular *reactive oxygen species* level was monitored by using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma), which crosses cell membranes and is hydrolyzed by intracellular nonspecific esterases to nonfluorescent DCFH. DCFH then is oxidized by *reactive oxygen species* to highly fluorescent compound DCF (Lebel et al., 1992). GES-1 cells were seeded into 96-well plates at a density of 5×10^4/ml. After overnight growth, cells were then treated with different concentrations of quercetin at 37 °C for 24 h. Cells were rinsed with PBS and then incubated with 100 μl of 5 μM DCFH-DA at 37 °C for 30 min. The fluorescence intensity of DCF was measured in a microplate-reader (Tecan Safire, Crailsheim, Germany) at excitation wavelength 485 nm and emission wavelength 530 nm. Then the cells were exposed to 300 μM H_2O_2 for 30 and 60 min at 37 °C and the fluorescence intensity of DCF was measured in a microplate-reader (Tecan Safire, Crailsheim, Germany) at excitation wavelength 485 nm and emission wavelength 538 nm. Data are presented as the fluorescence intensity ratios (*after* H_2O_2 *added*)/(*before* H_2O_2 *added*).

2.4. Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) measurements

$[Ca^{2+}]_i$ was determined with the Ca^{2+}-sensitive fluorescent dye Fura-2/AM (Sigma, St. Louis, MO) according to previous procedure with modifications (Sareen et al., 2007). GES-1 cells were seeded in 96-well plates at the density of 5×10^4/ml. After overnight growth, cells were treated with quercetin with 2% fetal bovine serum for 24 h. Then cells were rinsed with HBSS (or Ca^{2+}-free HBSS) and loaded with 2 μM Fura-2/AM in HBSS (or Ca^{2+}-free HBSS) containing 4% BSA at 37 °C for 30 min. Thereafter, cells were washed with HBSS or Ca^{2+}-free HBSS in the presence of 4% BSA once and exposed to 300 μM H_2O_2 or HBSS (or Ca^{2+}-free HBSS) for different incubation times. The fluorescence signal of $[Ca^{2+}]_i$ was determined in a microplate-reader (Tecan Safire, Crailsheim, Germany) at excitation wavelength of 340 nm or 380 nm and emission wavelength of 510 nm. Data are presented as the ratio of the fluorescence at 340 nm excitation to that at 380 nm excitation.

2.5. Measurement of mitochondrial inner membrane potential ($\Delta\Psi m$)

ΔΨm was monitored by applying the fluorescence dye JC-1 (Molecular Probes). For this purpose, GES-1 cells were seeded in 96-well plates at the density of 5×10^4/ml. After overnight growth, cells were incubated with quercetin at the concentrations of 25, 50 and 100 μM for 24 h. Cells were exposed to 400 μM H_2O_2 or PBS for 3 h, then the old medium was removed. Cells were then subjected to 100 μl of 10 μM JC-1 at 37 °C for 30 min. The medium was then discarded, and the cells were washed three times with PBS. The fluorescence intensity of JC-1 was measured in a microplate-reader (Tecan Safire, Crailsheim, Germany) at excitation wavelength of 485 nm or 550 nm and emission wavelength of 535 nm or 600 nm.

2.6. Real time RT-PCR

Cells were seeded in 6-well-plate at a density of 5×10^5 cells/well for approximately 16 h before drug was added. After added

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