Quercetin Alleviates Intestinal Oxidative Damage Induced by H$_2$O$_2$ via Modulation of GSH: In Vitro Screening and In Vivo Evaluation in a Colitis Model of Mice

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ABSTRACT: The gastrointestinal tract is exposed to pro-oxidants from food, host immune factors, and microbial pathogens, which may induce oxidative damage. Oxidative stress has been shown to play an important role in the onset of inflammatory bowel disease. This study aimed to use a novel model to evaluate the effects of a screened natural component and explore its possible mechanism. An in vitro oxidative stress Caco2 cell model induced by H$_2$O$_2$ was established using a real-time cellular analysis system and verified by addition of glutathione (GSH). A variety of plant components were chosen for the screening. Quercetin was the most effective phytocombination to alleviate the decreased cell index caused by H$_2$O$_2$ among the tested plant components. Furthermore, quercetin ameliorated dextran sulfate sodium salt (DSS)-induced colitis and further increased the serum GSH. The mechanism of quercetin protection was explored in Caco2. Reversed H$_2$O$_2$-induced cell damage and decreased reactive oxygen species and apoptosis ratio were observed in quercetin-treated cells. Also, quercetin increased expression of the glutamate-cysteine ligase catalytic subunit (GCLC), the enzyme of glutathione synthesis, and increased intracellular GSH concentration under H$_2$O$_2$ treatment. This effect was abolished by the GCLC inhibitor buthionine sulfoximine. These results indicated that quercetin can improve cell proliferation and increase intracellular GSH concentrations by upregulating transcription of GCLC to eliminate excessive reactive oxygen species (ROS). Increased extracellular H$_2$O$_2$ concentration induced by quercetin under oxidative stress was related to the inhibition of AQP3 and upregulation of NOX1/2, which may contribute to the observed protective effects of quercetin. Moreover, the novel H$_2$O$_2$-induced oxidative stress cell model based on the real-time cellular analysis system was an effective model to screen natural products to deal with intestinal oxidative damage and help accelerate the discovery of new drugs for inflammatory bowel disease (IBD).

INTRODUCTION

The intestinal epithelium serves as both a selective barrier for the absorption of nutrients and a protective barrier to prevent luminal antigens, microorganisms, and toxins from entering the internal environment.$^1$ The intestinal tract is exposed to pro-oxidants derived from ingested food constituents including iron, copper, H$_2$O$_2$, heme, lipid peroxides, and microbial pathogens. Thus, the gastrointestinal tract is a key source of reactive oxygen species (ROS).$^2$ In particular, the colon generates more endogenous ROS than the small intestine, and colonic antioxidant enzymes appear to be unable to reduce oxidative DNA damage in the presence of elevated ROS.$^3$ Oxidative stress is considered to be one of the etiologic factors involved in several symptoms of inflammatory bowel disease (IBD) like diarrhea and abdominal pain.$^4$ Activated neutrophils and macrophages contribute to the reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation, and the level of ROS can be correlated with the severity of inflammation in the colonic mucosa.$^5$ Disruption of the intestinal epithelial barrier contributes to the onset and acceleration of inflammation in IBD.$^6$ Due to the lack of a specific treatment and precise etiology for IBD, the goals of IBD therapy are to induce the remission of the symptoms, thus preventing the intestinal inflammatory process.$^7$ However, treatments for IBD using aminosalicylates and immunosuppressants have potentially serious side effects.$^7$ So there is a clear demand for safe and effective strategies for IBD.

However, most of the in vitro screening models focused on phagocytes such as RAW264.7 challenged by lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α) by determining the production of nitric oxide, proinflammatory mediators TNF-α, and interleukin-6 (IL-6).$^8,9$ However, IBD was characterized by both excessive inflammatory responses and...
subsequent loss of the epithelial barrier. The epithelial barrier controlled the passage of external bacteria, and the activation of immune response in turn exacerbated epithelial barrier dysfunction.10 Prolonged dextran sulfate sodium salt (DSS) treatment prevented healing and regeneration of the intestinal epithelium and led to chronic inflammation and lymphocyte infiltration.11 A single target on the phagocyte related to inflammation may not be comprehensive. Otherwise, an intact epithelial barrier and its regeneration were also important for the symptom alleviation of colitis and enhancing the phase of remission for colitis. Targeting these will be promising strategies for colitis recovery. Therefore, an effective model to simulate the epithelial cell under inflammation is needed.

Plant components such as flavonol (quercetin), no-flavonoid polyphenol (resveratrol), their glycoside forms rutin and polydatin, respectively, diterpene lactone (andrographolide), phenol (curcumin), and triterpene acid (ursolic acid) showed inflammatory, morphology modulation, and antioxidant properties in vitro or in vivo, which can be promising candidates for colitis therapy. (1) Plant components with anti-inflammatory function: Quercetin attenuated small intestinal mucosal damage from ischemia-reperfusion injury by inhibiting neutrophil infiltration in rats.12 Dietary rutin ameliorated DSS-induced colitis by suppressing the generation of proinflammatory cytokines.13 Resveratrol exhibited immune modulation function by regulating the Treg/Th17 balance, thus alleviating the DSS-induced colitis.14 (2) Plant components with morphology modulation function: Polydatin induced a transition from the proliferative morphology to cell-specific differentiated structures in human Caco2.15 (3) Plant components with antioxidant function: Andrographolide protected HepG2 cells from H2O2-induced cytotoxicity primarily by upregulation of Nr2f2/HO-1 via adenosine A2a receptor signaling.16 Curcumin was able to sustain spermatozoaa viability in the presence of ferrous ascorbate in an oxidative damage model in vitro.17 Ursolic acid induced uncoupling of oxidative phosphorylation in the heart mitochondria in a dose-dependent manner and significantly suppressed the H2O2 production in isolated mitochondria.18 Moreover, glutathione (GSH) was considered as one of the most abundant endogenous antioxidant molecules, which can directly or indirectly react with ROS.19 In addition, in a mouse model, dietary GSH had beneficial effects on acute signs of IBD.20 However, these plant components were not evaluated in the same condition and cannot be compared with each other. Therefore, it is essential to re-evaluate these natural products in a fast and effective model associated with epithelial barrier and cell proliferation.

In our study, we presented a cell model based on the xCELLigence system to simulate the oxidative stress circumstances in local inflammation during IBD. A variety of natural products were re-evaluated based on this cell model, and the most effective natural product was chosen for in vivo verification, and the possible mechanism was further explored.

### RESULTS

#### In Vitro Oxidative Stress Model Based on the xCELLigence System

Based on our previous studies, 1.5 mM H2O2 was applied to induce acute oxidative damage in Caco2. To verify the validity of the oxidative stress model for screening effective antioxidants, glutathione (GSH), an important inherent antioxidant molecule, was used as a positive control. Cells were allowed to grow for 24 h (time point A, green arrow) to synchronize the cell cycle and then were precultured with GSH for 18 h before H2O2 treatment (time point B, red arrow) (Figure 1a). At time point C (blue arrow), the culture medium with H2O2 was replaced by a medium with GSH only, allowing the cell to recover. GSH pretreatment not only alleviated the decreased CI caused by H2O2 during the stress phase (time point B to C) but also helped the damaged cell return to the normal status during the recovery phase (time point C and later). To confirm the alleviation effects of GSH, 2 h after addition of H2O2 cell viability was examined. The same protective effect of GSH was observed (Figure 1b). Same treatment processes including the cell attachment phase (phase I, from beginning to point A), the pretreatment phase (phase II, from point A to point B), and the oxidative stress phase (phase III, from point B to point C) were applied to explore the effective antioxidant in this study with GSH replaced by plant components.

#### Analysis of Kinetic Cellular Responses to Plant Components under Oxidative Stress

Proper concentration of each plant component was chosen based on preliminary cytotoxicity assays by real-time cell analysis (RTCA). As shown in Figure 2a, prolonged exposure of cells to H2O2 decreased the cell index at the 42nd hour and became stable after an additional 12 h of treatment. Then, 12.5–100 μM quercetin partially alleviated the decline in the cell index caused by H2O2 (Figure 2a). Similarly, 12.5–100 μM resveratrol partly reversed the decreased viability (Figure 2b).
Figure 2. Screening for effective antioxidants in the H\textsubscript{2}O\textsubscript{2}-Caco2 model by RTCA. Quercetin was examined for its antioxidant potential by RTCA (a) and viability (h), and its glycoside form rutin did not show any protective effect by RTCA (b) or cell viability assay (i). Andrographolide could slightly alleviate the decreased CI (c) and cell viability (j) induced by H\textsubscript{2}O\textsubscript{2}. Resveratrol increased CI (d) during the pretreatment phase, while its derivative polydatin did not (e), but neither of them showed any protective function. Curcumin (f) and ursolic acid (g) did not show any protective effects and even worse decreased the CI during the pretreatment phase. Mean without a common letter differs, \( P < 0.05. \)
Rutin (quercetin-3-O-rutinoside), a glycoside between quercetin and the disaccharide rutinose, did not show any protective effect against H2O2-induced decrease of CI (Figure 2b) or cell viability (Figure 2i). Moreover, 6.25 μM andrographolide slightly alleviated the decrease of CI (Figure 2c) and cell viability (Figure 2j). Since the changes of cell
viability were consistent with those of CI, RTCA was used for the following screening analysis of effective antioxidants. Resveratrol and its derivative polydatin (Reservatrol-3-β-D-glucoside) could not protect cells from H2O2, but only 100 μM resveratrol significantly enhanced the CI during the pretreatment phase, while polydatin did not (Figure 2d,e). Curcumin and ursolic acid did not exhibit any protective effects against H2O2 and even led to a sharp decline of CI during the pretreatment phase (Figure 2f,g). Among the plant components tested, quercetin showed excellent protective effects and was chosen for subsequent experiments.

Quercetin Alleviated the Decreased Relative Body Weight and Intestinal Injury in a Colitis Model Induced by DSS. The results demonstrated a significant reduction in the relative body weight in the DSS group, but dietary quercetin at 500 and 1500 ppm partly counteracted the DSS-induced body weight loss 5–6 days after DSS intake (Figure 3a). The reduced severity of intestinal inflammation by quercetin was evidenced by the recovered colon length (Figure 5).

Figure 5. (a) Quercetin (25 μM) partly reversed the proliferation inhibition by H2O2. Red arrows indicate the EdU positive cells. (b) Quercetin (25 μM) partly alleviated the early apoptosis induced by H2O2. Red circles indicate the early apoptosis ratio of cells. Ctr/Con, control group; Q, quercetin treatment; H, H2O2 treatment group; QH, cotreatment of H2O2 and quercetin.

Figure 6. Effects of quercetin on GSH abundance in Caco2 cells exposed to H2O2. (a) Quercetin alleviated the decrease of total GSH induced by H2O2. (b) Coincubation of quercetin and H2O2 increased the intracellular reduced GSH compared to the H2O2 treatment. (c) Quercetin and H2O2 alone or combined increased the expression of MRP1. (d) Quercetin increased the mRNA expression with or without H2O2. Mean values with different letters in the same picture are significantly different (P < 0.05).
3b). Furthermore, Figure 3c presents the results of histological evaluation of the colon. Significant histological injury such as mucosal erosions or lamina propria inflammatory cell infiltration was reduced by the quercetin treatment compared to the DSS treatment. Similarly, the histological pathological score was much lower in the quercetin treatment group compared to the DSS treatment (Figure 3d).

**Quercetin Alleviated the Increased Intestinal Permeability and Disordered Antioxidant Status in a Colitis Model Induced by DSS.**

Intestinal barrier loss was characterized by two probes: FITC-4 kDa dextran and rhodamine-70 kDa dextran, which can be used to probe leak and unrestricted pathways respectively. Increased intestinal permeability to 4 and 70 kDa dextran by DSS that occurred as a result of epithelial damage returned to the baseline level under the treatment of quercetin (Figure 4a,b). Serum GSH as an important antioxidant molecule was determined. DSS significantly increased the serum GSH, and quercetin (1000 ppm) further increased the GSH level (Figure 4c). In the liver, 500 ppm quercetin increased the activity of superoxide dismutase (SOD) and catalase (CAT) compared to the DSS treatment, but 1000 ppm quercetin further decreased the activity of CAT (Figure 4d,e).

**Quercetin Protected Caco2 Cells from Damage Induced by H2O2-Modulating Cell Proliferation and Apoptosis.** The protective effects for cell viability against H2O2 damage by quercetin were related to the increase of cell proliferation or a reduction of apoptosis. As Figure 5a,b demonstrates, H2O2 led to complete inhibition of cell proliferation ($p < 0.05$), which was partly attenuated by quercetin treatment. In addition, early apoptosis induced by H2O2 was significantly alleviated by quercetin (Figure 5b).

**Quercetin Protected Caco2 Cells from H2O2-Induced Oxidative Damage by Modulating Intracellular GSH.**

Total GSH was determined by commercial assay kits based on the DTNB method. The total GSH was significantly decreased by H2O2, whereas 25 μM quercetin alleviated the H2O2-mediated decrease of GSH (Figure 6a). H2O2 and quercetin coincubation significantly increased abundance of reduced GSH compared to the H2O2 group (Figure 6b). The mRNA expression of MRP1 (ATP binding cassette subfamily C member 1) was significantly increased in the Q, H, and QH groups compared to the control group (Figure 6c). Quercetin also significantly upregulated the transcription of glutathione reductase (GR) compared to the quercetin-free group with/without H2O2 (Figure 6d).

**Influence of GCLC Inhibitor on the Protective Function of Quercetin on Caco2 Cells Exposed to H2O2.**

To validate whether GSH synthesis played a critical role in the protective function of quercetin, BSO (GCLC inhibitor) and BCNU (GR inhibitor) were used. As shown in Figure 7a, quercetin reversed the decreased viability caused by H2O2. Quercetin-induced protection against H2O2 was partly abolished by BCNU treatment and completely eliminated with BSO or BSO + BCNU treatment. In Figure 7b, intracellular GSH was measured using the fluorescent probe mBCh. H2O2 significantly ($P < 0.05$) decreased intracellular GSH compared with the control, while quercetin reversed GSH concentrations to the normal status. When GCLC was inhibited by BSO, intracellular GSH in the QH group was decreased to the level of the H2O2-only treatment group. Coinhibition of GCLC and GR in the quercetin + H2O2 cotreatment group lead to a significant decrease of GSH compared to the QH treatment.
Furthermore, mRNA transcription and protein expression of the GCLC gene were measured by RT-PCR and western blot in triplicate (Figure 7c,d). H2O2 significantly repressed mRNA and protein expression of GCLC compared to the control group (P < 0.05). Quercetin reversed this effect, which was attenuated by BSO treatment.

Quercetin Restrained Intracellular ROS Generation but Increased Extracellular H2O2 of Caco2 Exposed to H2O2. To elucidate the protective function of quercetin on Caco2 cells, the changes of intracellular ROS and extracellular H2O2 concentrations were measured. As shown in Figure 8a, the ROS level in the H2O2 treatment group significantly (P < 0.05) increased compared to the control group, but this effect was reduced by quercetin pretreatment. Next, 5–10 μM AgNO3 significantly increased the cell viability compared to the control group and partly restrained the cell viability reversed by quercetin under oxidative stress (Figure 8b). However, cell viability in AgNO3 together with the H2O2 treatment was not significantly different from the H2O2 treatment (Figure 8b). Surprisingly, extracellular H2O2 concentration in the quercetin + H2O2 coinubcation treatment group (QH) was much higher than H2O2 concentration in the H2O2-only treatment group (H) (P < 0.05) (Figure 8c).

AgNO3 as a nonspecific aquaporin (AQP) inhibitor blocked H2O2 intake via AQP. AgNO3 further increased extracellular H2O2 concentration in the Ag + QH group compared to the QH group (Figure 8c). In addition, the initial H2O2 concentrations in the H2O2-treated culture medium (478 μM), H2O2 and quercetin coinubcation culture medium (493 μM) without cells, were much higher than the concentration of extracellular H2O2 with living cells after H2O2 treatment for 2 h with or without quercetin. No significant change of intracellular H2O2 concentrations was detected.

The expressions of AQP3 and NOX1/2 mRNA were examined (Figure 8d–f). Under oxidative stress, quercetin downregulated the transcription of AQP3 but upregulated NOX1/2 transcription levels compared to the H2O2 treatment group.

**DISCUSSION**

As one of the most proliferative tissues, the gastrointestinal epithelium self-renews every 4–5 days. Meanwhile, the intestine constitutes an essential barrier against harmful substances. Intact intracellular contacts between epithelial cells are essential to maintain a defense against harmful substances and pathogens from the external environment. Oxidative stress is clearly involved in inflammatory bowel disease (IBD) when inflammation occurs and could be a major factor contributing to tissue injury. In addition, hydrogen peroxide can be produced by monocytes, lymphocytes, and principally neutrophils coming from leukocyte infiltration, which is characteristic of IBD.

A cell-based screening assay, namely, the xCELLigence system, was used. The cells were continuously monitored in real time by this system, which produced specific time-/dose-dependent cell response profiles (TCRPs) under treatment with different biological active compounds. TCRP reflected the changes of cell statuses, including cellular morphology, adhesion, and growth, which are modulated by cellular interactions upon a variety of treatments. In humans, impaired H2O2 regulation in the intestine has been associated with early-onset inflammatory bowel disease (IBD) and colon...
cancer.28 As a stable ROS, H2O2 mediates extracellular and intracellular signals for cell growth, differentiation, and migration. Since intestinal epithelial cell growth and cellular interactions changed and excessive ROS was generated in the colonic tissue during colitis, H2O2-induced oxidative damage was used to simulate the oxidative stress circumstances, and cell responses were monitored by the xCELLigence system.

The cell index measured by the RTCA system is proportional to the number of adherent cells, inherent morphology, and adhesive characteristics of cells.23 The RTCA system is considered to be a promising tool for monitoring epithelial barrier function in situations with more physiological relevance26 and may provide a more comprehensive picture of cell changes in different circumstances. Thus, the oxidative stress cell model induced by H2O2 applied in the RTCA system was established to screen effective antioxidant plant components. GSH, an important naturally occurring cellular reductant and antioxidant molecule, was used to verify the validity of the model. In another study, GSH also decreased H2O2-mediated apoptosis in IEC-6 cells.29 Similarly, in our study, GSH alleviated the decreased CI and cell viability caused by H2O2. In addition, after oxidative damage for 2 h, the deprivation of H2O2 with continuous GS addition restored the CI back to the normal level. These results indicated that this model could effectively monitor the cellular responses under oxidative stress induced by H2O2 and would be effective for screening highly active antioxidants.

In our studies, several promising phytochemicals were chosen for the in vitro screening tests. Quercetin (flavonol) protected cells from oxidative stress, while its glycoside derivatives quercetin-3-O-rutinoside (rutin) did not show any protective effects. In situ perfusion in the rat intestine demonstrated that rutin was hardly absorbed compared with quercetin aglycone because rutin was easily digested not in the small intestine but rather in the large intestine by intestinal microbiota.30 Andrographolide (diterpene lactone) slightly reversed the decreased CI and cell viability. Similarly, andrographolide was reported to activate Nrf2, leading to its translocation to the nucleus and activation of heme oxygenase (HO-1), thus protecting HepG2 cells against H2O2-induced cell death.16 Resveratrol (Anthraquinone terpenoids, non-flavonoid polyphenols) increased epithelial expression of occluding and ZO1 in a dose-dependent manner and protected Caco2 from H2O2-induced oxidative damage via upregulation of HO-1.31 However, resveratrol also showed growth inhibitory and cell-cycle arrest effects on Caco2 cells.32 Polydatin (resveratrol-3-β-D-glucoside) had a stronger cytotoxicity than resveratrol in growing Caco2.32 However, along with the reduction in cell viability, resveratrol and polydatin changed the cell structure possibly by cytoskeleton rearrangements.33 As previously addressed, reduction in cell viability and changes in the cytoskeleton both contributed to the change of CI monitored by the RTCA system. In our experiments, resveratrol enhanced CI and polydatin did not, while neither of them protected cells from oxidative damage, and the reason might be that cells used were in the growing phase. Moreover, 25−50 μM curcumin (phenol) was supposed to be the most effective for maintaining spermatozoon viability under ferrous ascorbate-FeAA-induced oxidative damage. Treatment of Caco2 cells with curcumin resulted in concentration-dependent cell death with an EC50 at about 59 μM.34 Ursolic acid (triterpene acid) significantly decreased cell viability at a concentration higher than 50 μM and also showed a protective effect against H2O2 (75 μM)-induced DNA damage at 10 μM.34 A similar cytotoxicity of curcumin and ursolic acid on Caco2 was also observed in our in vitro study. However, the H2O2 used in our experiments was much higher, and more damaging oxidative stress was induced. Above all, among the plant components tested, quercetin showed excellent protective effects against H2O2 and was chosen for subsequent experiments.

IBDs are associated with oxidative stress, which may play a significant role in their etiologies.35 ROS and proinflammatory cytokines have a continuous implication in the progression of ulcer colitis.36 Different from superoxide anion, which is highly reactive and unstable, H2O2 can freely diffuse across cells and oxidize compounds located further.36 Quercetin, known as one of the most abundant dietary flavonoids, is ubiquitously present in food including fruits, vegetables, and teas.37 The half-life of quercetin metabolites is from 11 to 28 h, which suggests that with repeated quercetin supplementation, a considerable plasma level can be attained.37 Quercetin possesses the key structural features for effective free radical scavenging and has been observed to be a powerful free radical scavenger in vitro.38 Quercetin was reported to ameliorate T-cell-mediated colitis in Rag1−/− mice partly by modulating the function of macrophages via HO-1.39 However, in another colitis model, dietary 0.1% rutin other than quercetin attenuated DSS (5%)-induced body weight loss and shortening of the colorectum and improved histological scores probably by suppressing the induction of proinflammatory cytokines.35 In the DSS (3%)-induced colitis model, dietary quercetin or mixture including quercetin and quercetin monoglycoside supplementation both counteracted the decreased body weight gain and improved the oxidative stress biomarker such as GSH, myeloperoxidase, and malonaldehyde.40 In our experiments, quercetin partly alleviated the body weight loss induced by DSS. Histological changes in the colon including tissue damage and lamina propria inflammatory cell infiltration were typical results obtained after the DSS colitis protocol.22 Compared to the DSS group, quercetin significantly counteracted the histological change of DSS-induced colitis. Increased intestinal permeability derived from dysfunction of the intestinal barrier is an important characteristic symptom in the pathophysiology of IBD.4 Quercetin alleviated the increased intestinal permeability evaluated by increased FITC-4 kDa and rhodamine B 70 kDa. Quercetin was able to rapidly increase transepithelial electrical resistance peaking at 6 h in Caco2 and elevated the ZO-2, occludin, and claudin-1 in the actin cytoskeleton without increasing their respective whole-cell levels.41 Excessive ROS lead to cytoskeletal protein damages and finally disrupted the intestinal barrier.32 GSH was supposed to be a key determinant in the elimination of peroxides by the intestine.42 However, in our experiments, DSS treatment increased serum GSH, which may be a result of the activated inherent antioxidant system. In addition, quercetin further increased the serum GSH to enhance the antioxidative status. Quercetin-S′-sulfonic acid sodium salt at the concentration of 50 μM exhibited the highest SOD activity in extracorporeal liver perfusion and was higher than the values obtained in 10 μM quercetin.43 In our experiment, only 500 ppm dietary quercetin increased the activity of liver SOD. Inhibition of pure catalase or cellular catalase from K562 cells by flavonoids was examined, and quercetin was the second or the first flavonoid exhibiting inhibition efficiency among myrecetin, kaempferol, lutrolin, and apigenin.45 Similarly, the
inhibition of quercetin on catalase was observed in 1000 ppm quercetin rather than in 500 ppm quercetin. Above all, in our study, quercetin partly alleviated the DSS-induced colitis with improved intestinal permeability and serum GSH. Based on the changes induced by DSS and the improvements achieved by quercetin, GSH was supposed to be a promising target to reveal the protective effects of quercetin on intestinal oxidative damage.

According to the in vitro tests (Figure 2a,b), 25 μM quercetin was chosen for this study. The change of cell viability under oxidative stress was closely related to proliferation and apoptosis. In a study of cardiac regeneration, EdU could rapidly and sensitively label proliferating cells in developmental and pathological states in vitro and in vivo. The EdU test data demonstrated that H2O2 completely inhibited the proliferation of Caco2 cells, while quercetin slightly alleviated the inhibiting effect of H2O2. Similar results were observed on IPEC-J2.47 H2O2 significantly increased the ratio of apoptotic cells, while quercetin improved the cell apoptosis status. Thus, quercetin ameliorated the decrease in cell viability induced by H2O2 through the enhancement of cell proliferation and modulation of the apoptosis status.

Based on the protective effects against H2O2-induced oxidative damages in Caco2 and improvement of serum GSH of quercetin in the DSS-induced colitis model, we studied whether GSH is a key factor for the protective function of quercetin against H2O2. H2O2 decreased total intracellular GSH and reduced GSH, but both were alleviated by quercetin (Figure 6a,b). The transporter of GSH was also measured in this study. MRP1 was exclusively responsible for GSSG export, especially under oxidative stress. Another enzyme, GR, was able to reduce glutathione disulfide (GSSG) to the sulfhydryl form reduced GSH, by an NADPH-dependent mechanism, which keeps intracellular reduced GSH in high concentration. In our study, both H and QH treatment increased the mRNA expression of MRP1 compared to the control group, but no significant difference was observed between H and QH treatments. However, the QH treatment significantly increased the expression of GR compared to the H treatment group, which was responsible for the higher concentration of reduced GSH in the QH treatment group. There were two important steps in the biosynthesis of GSH, with two enzymes involved: (1) γ-glutamyl-cysteine synthase (GCL) that links glutamate with cysteine in an ATP-dependent reaction and (2) glutathione synthetase (GS) that reduces glutathione to γ-glutamyl-cysteine and glycine in a second ATP-dependent reaction.49 The GCL linkage was the rate-limiting step in the biosynthesis of GSH. The key enzyme GCL is composed of two subunits: a catalytic subunit, glutamate-cysteine ligase catalytic subunit (GCLC), and a modulatory subunit, glutamate-cysteine ligase modifier (GCLM).50 In Figure 7a,b, the GCLC inhibitor or coinhibition of GCLC and GR both abolished the protective effects of quercetin on cell viability and intracellular GSH. The GCLC inhibitor significantly downregulated the mRNA expression of GCLC, which was upregulated by quercetin under H2O2 treatment. Quercetin alleviated the H2O2-induced decrease of GCLC, which was also confirmed by western blot (Figure 7c). These results indicated that the upregulation of GCLC by quercetin played a key role in the protective functions under oxidative stress in Caco2 cells. The upregulation of quercetin on GCLC was also observed in another study.50 Overall, upregulation of both GR and GCLC contributed to the protective effect of quercetin from oxidative stress induced by H2O2.

ROS, including H2O2, was generated as byproducts of normal metabolism in biological systems.51 Low levels of ROS were essential for cell differentiation, apoptosis, and function as secondary messengers, but excessive ROS had detrimental effects on cellular components including DNA, protein, and lipids. Unlike the highly reactive superoxide anion, H2O2 can convert to a hydroxyl radical and freely diffuse across cell membranes. ROS generation increased approximately 10-fold when Caco2 cells were challenged with H2O2 (22.5) versus the control group (2.53).52 Similarly, in our study, ROS was significantly increased in cells treated by H2O2, but quercetin restored intracellular ROS to the control level. Chen et al. also reported that 5 μg/mL quercetin was able to decrease ROS concentration in IPEC-J2 cells after exposure to 1 mM H2O2.47

However, in our study, quercetin increased the extracellular H2O2 concentration approximately 2.5-fold in the culture media compared to the H2O2-treated group yet still alleviated the H2O2-induced oxidative damage at the same time. As antioxidant proteins in the cell like glutathione peroxidases could rapidly eliminate free cellular H2O2, the membrane permeability of cells is the primary rate-limiting step for intracellular increase of H2O2.53 AQP3 located on the cell membrane was a water-, glycerol-, and H2O2-transporting protein implicated in various cellular functions.54 AQP3 facilitated the uptake of H2O2 into mammalian cells and mediated intracellular signaling.55 When exposed to extracellular H2O2, cystolic H2O2 increased much faster in cells expressing AQP3 compared with cells in which AQP3 was inhibited by AgNO3.56 In leukemia cells, AQP inhibition caused a decrease in intracellular ROS accumulation both when H2O2 was produced by Nox enzymes and when it was exogenously added.57 In our study, H2O2 treatment upregulated the mRNA expression of AQP3 compared to the control, which was reversed by quercetin. Reduced transcription of AQP3 in the quercetin-H2O2 coinubation group (QH) led to less uptake of H2O2 into the cell, which can be rapidly eliminated by intracellular antioxidant proteins. Consistent with this, AgNO3 (an inhibitor of AQP3) further increased the extracellular H2O2 concentration in the QH group compared to H2O2 treatment. Both quercetin and AgNO3 increased H2O2 in the medium and could have synergistic effects in enhancing the increase of extracellular H2O2. NOX1, highly expressed in the colon, is engaged in the generation of extracellular H2O2 in the mucosa, and AQP3 can facilitate the entry of native NOX-dependent H2O2 into the cell.58 In our study, the mRNA expression of NOX1 was upregulated in the H2O2 treatment group compared to the control group, which was further increased by coinubation of quercetin and H2O2. Therefore, quercetin may increase extracellular H2O2 by inhibiting the expression of AQP3, which facilitated the entry of H2O2 into the cell, and upregulating NOX1, which increased the generation of extracellular H2O2, which may also be involved in the protection of quercetin against H2O2. In addition, epithelial generation of H2O2 has been shown to be an important signal for wound repair, part of the host response to gut infection and involved in microbial clearance.

CONCLUSIONS
In our studies, quercetin was able to ameliorate DSS-induced colitis in mice and increase intracellular GSH to eliminate...
excessive ROS induced by H$_2$O$_2$ by upregulating transcription of GCLC and GR, therefore improving the proliferation and apoptosis status in Caco2 cells under oxidative stress. These results indicated that quercetin was a promising candidate for the therapy of IBD, and the oxidative stress cell model based on the xCELLigence system was an effective tool for screening plant components to deal with intestinal oxidative damage and IBD.

**METHODS**

**Materials.** Dulbecco’s modified Eagle’s medium (DMEM), trypsin with/without ethylenediaminetetraacetic acid (EDTA), penicillin−streptomycin, and sterile phosphate-buffered saline (PBS) were obtained from Solarbio Co., Ltd. (Beijing, China). Reduced GSH, quercetin, rutin, andrographolide, resveratrol, polydatin, curcumin, and ursolic acid were obtained from Solarbio Co., Ltd. (Beijing, China), and all of the plant components are standards with purity greater than 99%. Fetal bovine serum (FBS) was purchased from Gibico (Gaithersburg, MD). Quercetin stock (25 mM) was dissolved in dimethyl sulfoxide (DMSO) and preserved under $-20 \, ^\circ C$. Dextran sulfate sodium salt (DSS, 36,000−50,000 Da) was purchased from MP Biomedicals.

**Cell Culture and Treatment.** Caco2 cells were purchased from the Institute of Animal Science of CAAS (Beijing, China). Caco2 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin, and streptomycin at 37°C in humidified air containing 5% CO$_2$.

Caco2 cells (2 x 10$^5$/well) grown for 24−48 h were allowed to attach to the culture plate before being pretreated by quercetin overnight. In the case of no inhibitors, cells were treated by 1.5 mM H$_2$O$_2$ in DMEM without FBS for 2 h. In the case of inhibitors, cells were treated with 1 M buthionine sulfoximine (BSO) for 6 h for GCLC inhibitor and 50 μM 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) for 1 h for GSH reductase inhibition after the pretreatment of quercetin and then treated by H$_2$O$_2$ for 2 h to induce oxidative stress.

**Mice and General Experimental Procedures.** A total of 72 male C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The animals were kept in a Laboratory Animal Platform of China Agricultural University SPF environment. All animals were raised under standard plastic cages at 21−24 °C room temperature with a 12/12 h light/dark cycle and free access to sterile tap water and food. Animal care and handling procedures were approved by China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Committee (approval number: AW04129102-2-1).

After 4 days of adaptation, mice were randomly assigned to six treatments, including the control group (Ctr), DSS-induced colitis group (DSS), and DSS-induced colitis supplemented with 100 ppm quercetin (DQ100), 500 ppm quercetin (DQ500), 1000 ppm quercetin (DQ1000), and 1500 ppm quercetin (DQ1500). Mice in the Ctr and DSS groups were fed standard mice Chow pellets, and mice in the DQ100, DQ500, DQ1000, and DQ1500 groups were fed standard mice Chow pellets supplemented with 100, 500, 1000, and 1500 ppm quercetin. Each treatment contained 12 mice with four mice per cage. After being pretreated with dietary quercetin in different concentrations for one week, the control group was given sterile fresh tap water ad libitum. In the next five groups, mice received different concentrations of quercetin with 3% (m/V) DSS added to the tap water for 6 days to induce colitis. The body weight of each mouse was recorded every day.

At the end of the experiment after 6 days to induce colitis, six mice from each group were killed by cervical dislocation, and blood was gained from the eye. The colon was removed from the abdominal cavity, and the length of the colon was measured. Three specimens were randomly selected for histopathological analysis by a pathologist (who had no knowledge of the experimental protocol) according to the scoring system for inflammation-associated histological changes in the colon. Briefly, tissue damage scores were assessed as follows: grade 0, none; grade 1, isolated focal epithelial damage; grade 2, mucosal erosions and ulcerations; and grade 3, extensive damage into the bowel wall. Lamina propria inflammatory cell infiltration was scored as follows: grade 0, infrequent; grade 1, increased, some neutrophils; grade 2, submucosal presence of inflammatory cell clusters; and grade 3, transmural cell infiltration.

**Measurement of Intestinal Permeability.** Mice were fasted without food but allowed water for 3 h. Mice were gavaged with 150 μL of 80 mg/mL 4 kDa FITCdextran and 40 mg/mL rhodamine B −70 kDa. After 3 h (continuing the food fast but allowing water), blood was collected and then the serum and distilled water were diluted in a ratio of 1:4. Next, 100 μL/well was added in a black plate for fluorescence with fluorescein isothiocyanate (FITC) (485 excitation/528 emission) and rhodamine B (565 excitation/590 emission).

**Measurement of Cell Viability and xCELLigence System.** Cell viability was measured using a CCK-8 kit according to the manufacturer’s instructions (Dojindo, Kumamoto, Japan). Briefly, Caco2 cells were seeded in a 96-well plate at 2 x 10$^4$ cell per well and cultured overnight. Cells were pretreated with different concentrations of quercetin for 18 h after plate attachment. Cells were then treated by H$_2$O$_2$ for 2 h, with or without use of the inhibitor. The culture medium was then replaced by 200 μL of DMEM supplemented with 10% CCK-8 per well and cultured at 37°C for 2 h. Afterward, the absorbance was measured at 450 nm on a Microplate Reader (Spectra Max i3x, Molecular Devices).

The xCELLigence system, a real-time cellular analysis system also known as RTCA (Roche Applied Science), was used to monitor the change of Caco2 cells treated by different concentrations of quercetin. This system used microelectronic plates with gold microelectrode arrays at the bottom of each well to monitor the cellular status every 10 min. In each well, low AC voltage produced an electric field between the electrodes that can be impeded by the adherent cells. The extent of the impedance change (namely, the cell index, CI) was proportional to the number of adherent cells and the inherent morphology and adhesive characteristics of the cells.

“Normalized cell index” at a certain time point was acquired by dividing the CI value by the value at a reference time point.

**Apoptosis Assay: Flow Cytometry Assay.** Caco2 cells (2 x 10$^5$/well) were seeded in a six-well plate, grown overnight, and then pretreated with quercetin before treatment with H$_2$O$_2$ for 2 h. Cell apoptosis was evaluated by flow cytometry with an Annexin V-FITC/PI apoptosis detection kit (Beyotime biotechnology Co., Beijing, China) following the manufacturer’s instructions. Briefly, cells after treatment were collected by trypsinization and washed with PBS. Cells were then resuspended in 250 μL of binding buffer with Annexin V-FITC/PI and incubated for 15 min. Apoptosis was analyzed by CytoFLEX (Beckman Coulter).
Table 1. Primer for RT-PCR Amplification

| gene | forward primer | reverse primer | note  |
|------|----------------|----------------|-------|
| GCLC | GTTCTTGAACCTCTGCAAGAGAAG | ATGGAGATGGTGTATTCTTGTCCT | NM_001498.4 |
| β-actin | TCACCCAACTGTCGCCAATCTACGA | TCGGTGAGGATCTTCATGAGGTA | NM_001101.5 |

**Cell Proliferation Assay.** Cell proliferation status was determined by the EdU assay using a Cell-Light EdU Apollo+488 In Vitro Kit (RiboBio Co., Guangzhou, China). Cell staining slides in a six-well plate were prepared as prescribed by the manufacturer’s protocol and observed by laser-scanning confocal microscopy (Leica TCS SP8, Germany).

**Determination of ROS and Hydrogen Peroxide.** ROS concentration was estimated using the ROS fluorescent-probe DCFH-DA (Solarbio, Beijing, China). Briefly, after incubation with quercetin, the Caco2 cells in a six-well plate were loaded with DCFH-DA according to the manufacturer’s protocol and then treated with H2O2 for 2 h. Afterward, the intracellular ROS level was measured by flow cytometry. A hydrogen peroxide assay kit (Beyotime biotechnology Co., Beijing, China) was used to measure extracellular H2O2 concentration in the culture medium according to the product protocol based on the reaction between ferrous ion and hydrogen peroxide.

**Measurement of GSH/GSSG and Antioxidant Enzyme.** Caco2 cells (2 × 106/well) were seeded in a six-well plate for measurement of GSH based on the DNTB method and the antioxidant enzyme and in a 96-well plate for the detection of total intracellular GSH by fluorescence probe mBCI (Monochlorobimane), grown overnight, and then pretreated with quercetin before treatment with H2O2 for 2 h. Two methods were used to measure the intracellular GSH levels of Caco2 in this study. (1) DNTB method: The GSH/glutathione disulfide (GSSG) ratio was measured by a commercial GSH and GSSG assay kit (Beyotime biotechnology Co., Beijing, China) based on the reaction between GSH and DNTB. (2) mBCI method: The fluorescent reagent mBCI was used to measure intracellular GSH levels of Caco2 cells, according to Kim and Jang.21 Briefly, when catalyzed by glutathione S-transferase, mBCI becomes fluorescent upon conjugation to GSH. This fluorescent probe can directly detect the GSH level in living cells. After incubating with 50 μM mBCI for 30 min, the fluorescence intensity was measured with an excitation wavelength at 380 nm and emission wavelength at 465 nm on a microplate reader (Molecular Devices SpectraMax i3x).

Superoxide dismutase (SOD) and catalase (CAT) were analyzed by a commercial kit (Nanjing Jiancheng Bioengineering Institute, China). Briefly, the activity of CAT was determined based on the reaction between ammonium molybdate and hydrogen peroxide under the catalytic action of catalase. The activity of SOD was detected by the WST method based on the reaction between SOD in the sample and the peroxide anion produced by xanthine oxidase. In the tissue homogenates, CAT and SOD were expressed as units per milligram of protein. Protein concentrations in the supernatant were assayed by the Pierce BCA Protein Assay kit (Thermo scientific).

**Measurement of mRNA Transcription.** Briefly, cells seeded in a six-well plate after treatments were collected. Total RNA extraction was carried out by an Easest Super Total RNA Extraction kit (Peomaga Co., Shanghai, China). RNA quantity was measured by Nanodrop at 260 and 280 nm. Then, the total RNA was reverse-transcribed into cDNA by a PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time) (Takara, Japan), and gene expression was determined by SYBR Premix Ex Taq (Ti RNaseH Plus, Takara, Japan) according to the manufacturer’s protocol. GCLC and β-actin primers are reported in Table 1, and 2−ΔΔCT was calculated to express the GCLC expression level.

**Western Blot Assays.** Briefly, cells seeded in a six-well plate after treatments were washed with ice-cold PBS and lysed in RIPA lysis buffer with protease inhibitor for 15 min on ice. Cell lysates were analyzed via a 12−15% SDS/PAGE gel and transferred to a PVDF membrane electrophoretically. After blocking with 5% skim milk, the blots were incubated by a primary antibody (GCLC, from Sangon Biotech; β-actin, from Sigma) overnight at 4°C, followed by a corresponding horse anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody, goat antimouse, or goat anti-rabbit (Sangon Biotech) for 1 h. Immune complexes were visualized with an ECL kit (Beyotime, China) according to the protocol. Signal intensity was quantified using a Tannon image analysis system (Tannon, China), and results were normalized to the signal intensity of β-actin.

**Statistical Analysis.** Data were expressed as the mean ± standard deviation. Differences between groups were evaluated by one-way analysis of variance (ANOVA) and Duncan’s multiple-range tests analyzed with SPSS17.0 (IBM, Armonk, NY). P < 0.05 was considered a statistically significant difference.

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Notes
The authors declare no competing financial interest.

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