Animal Nutriomics

cambridge.org/anr

Roles of MAPK and Nrf2 signaling pathways in quercetin alleviating redox imbalance induced by hydrogen peroxide in mammary epithelial cells

Yongxin Li[1], Ning Han[1], Pengfei Hou[1], Feng-Qi Zhao[2] and Hongyun Liu[1]

[1]College of Animal Sciences, Zhejiang University, Hangzhou, People's Republic of China and [2]Department of Animal and Veterinary Sciences, University of Vermont, Burlington, VT, USA

Research Article

Cite this article: Li Y, Han N, Hou P, Zhao FQ, Liu H (2024) Roles of MAPK and Nrf2 signaling pathways in quercetin alleviating redox imbalance induced by hydrogen peroxide in mammary epithelial cells. *Animal Nutriomics* **1**, e1, 1–10. https://doi.org/10.1017/anr.2024.2

Received: 21 December 2023
Revised: 2 February 2024
Accepted: 4 February 2024

Keywords:
quercetin; mammary epithelial cell; oxidative stress; MAPK signaling pathway; Nrf2 signaling pathway

Corresponding author: Hongyun Liu; Email: hyliu@zju.edu.cn

Abstract

Oxidative stress is a risk factor for mammary health, resulting in decreased milk yield and milk quality. Application of exogenous bioactive compounds has been a research focus of antioxidation of animals in the mammary gland. Quercetin is a flavonoid extracted from vegetables, fruits and tea and has been shown to have a variety of biological activities, but the effect of quercetin on redox imbalance in mammary epithelial cells is unclear. In this study, cells of HC11, a mouse mammary epithelial cell line, were treated with quercetin, and the effects and molecular mechanisms of quercetin protection on hydrogen peroxide-induced oxidative stress were studied. Results showed that 20 μM quercetin attenuated hydrogen peroxide-induced lactate dehydrogenase release and reactive oxygen species (ROS) accumulation and alleviated the reduction of cell viability and antioxidant capacity. Quercetin significantly restored the activation of mitogen-activated protein kinase (MAPK) and nuclear factor E2-related factor 2 (Nrf2) pathways induced by hydrogen peroxide. Importantly, the inhibitors of p38 MAPK and extracellular regulated protein pathways affected the activation of Nrf2 pathway. All inhibitors of MAPK and Nrf2 pathways reduced the protective effects of quercetin on cell proliferation, the activity of catalase and the expression of glutamate-cysteine ligase modifier subunit. Meanwhile, the effects of quercetin on the production of ROS and expression of glutamate/cystine reverse transporter light chain were mainly dependent on Nrf2 pathway. In summary, the protective effect of quercetin in mammary epithelial cells was mediated via MAPK and Nrf2 pathways.

Introduction

Milk consumption is critical for the survival, growth and development of mammal neonates, and mammary health is important for milk production (Garwolinska et al. 2018; Li et al. 2019). Oxidative stress is a common factor that impairs mammary health and the function of milk synthesis (Li et al. 2019; Spitzer et al. 2020). Oxidative stress occurs when the scavenging capacity of reactive oxidants is lower than their production, resulting in excessive accumulation of oxidative damages in the mammary gland which leads to decreased milk production and milk quality (Jakubczyk et al. 2020; Spitzer et al. 2020). Studies showed that some nature substances have great potential to combat oxidative stress (Xu et al. 2019, 2021). Quercetin, a kind of flavonoid substance found in vegetables, fruits, tea, wine and other foods, is one of examples (Polera et al. 2019; Xu et al. 2019). In drug research and development, quercetin has been found to have great potential for its antioxidant activity (Song et al. 2020; Xu et al. 2019). Studies showed that quercetin improved the expression of antioxidant enzymes in lipopolysaccharide (LPS) -challenged human aortic endothelial cells (Li et al. 2016), and quercetin protected human umbilical vein endothelial cells against H_2O_2-induced oxidative damages (Tian et al. 2019), suggesting its antioxidant capacity in cardiovascular diseases. However, whether quercetin has protective effects on oxidative stress in the mammary gland has not been well studied.

It is believed that nuclear factor E2-related factor 2 (Nrf2) signaling pathway is the main regulatory pathway of cellular antioxidant defense system (Shaw and Chattopadhyay 2020). When oxidative stress occurs, Nrf2 pathway is activated and regulates the activity of antioxidant enzymes and the expression of stress-resistant proteins to maintain redox homeostasis (Jin et al. 2016; Shaw and Chattopadhyay 2020). Severe oxidative stress leads to cell proliferation obstruction, autophagy and apoptosis. The mitogen-activated protein kinase (MAPK) pathway plays an important role in cell survival and death and thus may be closely related to oxidative stress (Kumar et al. 2021; Liu et al. 2020a; Luo et al. 2019). The MAPK family mainly consists of three subtypes: p38 MAPK, extracellular regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) (Jin et al. 2016). It has been

reported that three subtypes of MAPK pathway had positive effects on the redox imbalance in melanocytes and sertoli cells (Li et al. 2020a; Liu et al. 2020a). However, it is not clear whether quercetin alleviates oxidative stress via Nrf2 and MAPK pathways in the mammary gland. Therefore, the aim of this study was to investigate the effect and underlying molecular mechanisms of quercetin against redox imbalance in mammary epithelial cells. Our study provided evidence to support the development and utilization of quercetin as an antioxidant additive in lactating animals.

Materials and methods

Reagent

Quercetin (Q4951) was purchased from Sigma Chemical (St. Louis, MO, USA) with the purity ≥95%. Hydrogen peroxide (H_2O_2, 323381-500 mL), bovine insulin (I6634) and epidermal growth factor (E4127) were also purchased from Sigma Chemical. Fetal bovine serum (No. 900-108) was purchased from Gemini Bio (Calabasas, California, USA). Nrf2 inhibitor (ML385), p38 MAPK inhibitor (SB203580), ERK inhibitor (PD98059) and JNK inhibitor (SP600125) were purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture

The HC11 mammary epithelial cells, derived from female mouse mammary gland, were obtained from American type culture collection (ATCC) (Manassas, Virginia, USA; CRL-3062) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, bovine insulin (5 μg/mL), epidermal growth factor (10 ng/mL), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) in a humid incubator with 5% CO_2 and 95% air at 37°C.

Cell treatment

After HC11 cells adhered to the 96-well plates and grew to 40% confluence, the culture medium was changed to serum-free medium added with H_2O_2 (50–1000 μM) or serum-containing medium added with quercetin (5–25 μM) for 24 h to determine the changes of cell proliferation and lactate dehydrogenase (LDH) release. The powder of quercetin was dissolved in dimethyl sulfoxide (DMSO) (D2650, Sigma) to obtain 25 mM and 20 mM storage solution, respectively, based on the highest concentrations required for individual experiment. The inhibitor was dissolved in DMSO to produce a storage solution of 1000 times the concentration of the working solution. At each use, the storage solution of quercetin and inhibitors were diluted with the medium used in the experiment. In experiments with quercetin treatment, 0.1% DMSO was added in the control group. In experiments, after the HC11 cells adhered to the 6-well plates and grew to 40% confluence, HC11 cells were pretreated with 20 μM quercetin for 2 h, followed by treatment of 100 μM H_2O_2 for 24 h to determine the effects of quercetin pretreatment on H_2O_2-induced redox imbalance and the activation of MAPK and Nrf2 pathways. In the experiments with signaling molecule inhibitors, HC11 cells were pretreated with an individual inhibitor for 1 h, and then treated with 20 μM quercetin for 2 h, followed by treatment with 100 μM H_2O_2 for 24 h.

Cell viability and cytotoxicity assay

Cell viability was quantified by Cell Counting Kit-8 (Beyotime, Shanghai, China). The 96-well plates were inoculated with about 8000 cells/well. After HC11 cells adhered and grew to 40% confluence, the cell treatments were carried out as described above. The CCK8 solution was added to each well and incubated for 1 h, and the absorbance at 450 nm was detected by microplate reader (Molecular Devices, California, USA). The cytotoxicity of cells was determined by LDH Cytotoxicity Assay Kit (Beyotime). When 1 h before the predetermined time reached, LDH releasing reagent was added to the "sample maximum enzyme activity control well." Then, the HC11 cells were cultured for 1 h followed by centrifuging at 400 × g for 5 min. A 90 μL volume of supernatant/each well was transferred to a new 96-well plate, and the absorbance at 490 nm was measured by microplate reader (Molecular Devices). In the cell viability and cytotoxicity experiments, six wells were used for one treatment in each experiment, and the experiment was conducted three times independently.

Cellular reactive oxygen species (ROS) assay

The production of cellular ROS was determined by DCFDA/H2DCFDA Cellular ROS Assay Kit (ab113851) purchased from Abcam (Waltham, MA, USA) using flow cytometry (BD FACSVerse™, BD Biosciences, CA, USA) with BD FAC Suite software (BD Biosciences). According to the manufactures' instructions, HC11 cells were cultured and treated with 100 μM H_2O_2 for 4 h. Then, the cells were prepared into a single cell suspension and incubated in 10 μM 2',7'–dichlorofluorescin diacetate (DCFDA) working solution at 37°C for 30 min in dark. After that, the cells were gently blowing with a pipette to ensure that single cells were suspended, and then 10,000 cells were collected and analyzed in each group by flow cytometry. The excitation wavelength of fluorescence was 485 nm, and the emission wavelength was 535 nm. The fluorescein Isothiocyanate-protein A (FITC-A) mean value was used to analyze the differences between groups.

Assay of total antioxidant capacity (T-AOC), catalase (CAT) and superoxide dismutase (SOD) enzyme activities

The T-AOC was defined as the ratio of reducing efficiency of the samples to the reducing efficiency of Trolox which determined by Total Antioxidant Capacity Assay Kit. The assays for CAT activity, SOD activity and protein concentration were performed using the Catalase Assay Kit, Total Superoxide Dismutase Assay Kit with WST-8 and BCA Assay Kit (Beyotime), respectively, according to the manufactures' instructions. The activities of CAT and SOD enzymes and T-AOC were normalized by the protein concentration of the samples.

Western blot analysis

Cells were lysed using Radio Immunoprecipitation Assay (RIPA) lysate buffer with 1 mM phenylmethanesulfonyl fluoride (Beyotime). The lysates were centrifuged at 12,000 × g for 10 min, and the supernatants were collected. The protein concentration was determined using BCA Assay Kit (Beyotime). The western blot analysis used 20 μg protein per well. All protein samples were used within 1 week after extraction. Other procedures of western blot were performed according to the previous description (Chen et al. 2020). Primary antibodies used for western blot were all rabbit anti-mouse antibodies which obtained from Abcam, HuaAn Biotechnology (Hangzhou, China) and Cell Signaling Technology (MA, USA). More information about the antibodies was listed in

https://doi.org/10.1017/anr.2024.2 Published online by Cambridge University Press

Table S1, and the secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (Bioker, Hangzhou, China). The Super ECL Detection Reagent (Bioker) was used for chemiluminescence using an imager from Shanghai CLINX Science Instruments Co., Ltd (Shanghai, China). The relative intensity of bands was quantified using Fusion FX software (Vilber, France) and normalized by reference protein β-actin in the same sample.

Statistical analysis

Data were presented as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison was carried out to determine the differences between treatments using IBM SPSS Statistics 19 (IBM, Armonk, NY, USA). $P < 0.05$ was considered statistically significant. Graphing was carried out using GraphPad Prism Software version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Effects of quercetin on H_2O_2-induced changes in cell viability and LDH release in HC11 cells

Cell viability was significantly reduced by 50–1000 μM H_2O_2, whereas 100–1000 μM H_2O_2 significantly increased LDH release in HC11 cells (Fig. 1A–B). The cell viability was reduced to about 60% of the control group, and LDH release was increased by about 25% when the cells were treated with 100 μM H_2O_2 for 24 h (Fig. 1A–B). Thus, treatment of cells with 100 μM H_2O_2 for 24 h was used as the cell injury model for subsequent experiments. Treatment of cells with 5–20 μM quercetin alone did not affect the cell viability, whereas 25 μM quercetin significantly reduced

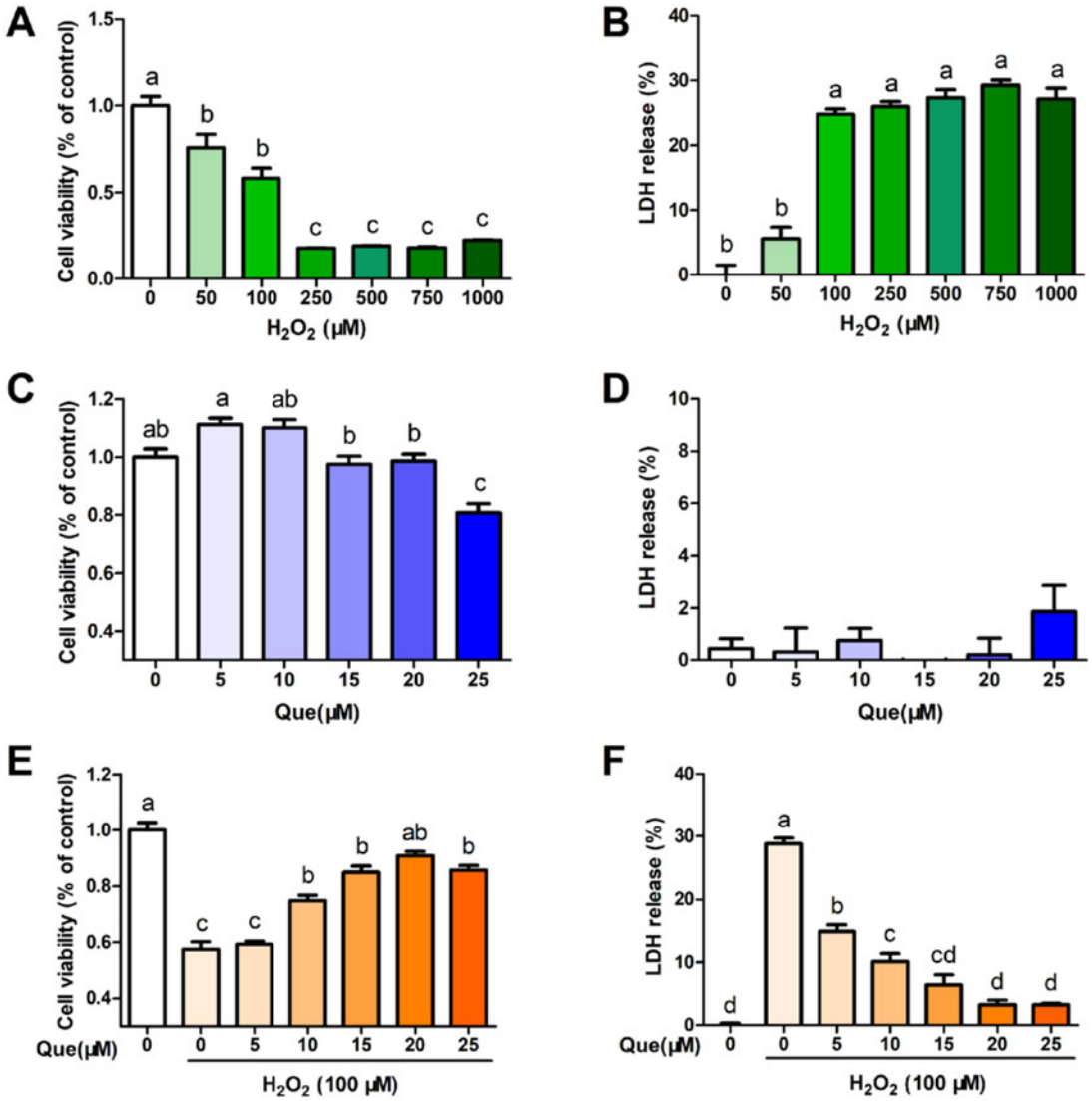

Figure 1. Effects of different concentrations of hydrogen peroxide (H_2O_2) and quercetin on cell viability and lactate dehydrogenase (LDH) release in HC11 cells. (A) Cell viability and (B) LDH release in HC11 cells after 24 h treatment with different concentrations of H_2O_2 (0, 50, 100, 250, 500, 750 and 1000 μM). (C) Cell viability and (D) LDH release in HC11 cells after 24 h treatment with different concentrations of quercetin (0, 5, 10, 15, 20 and 25 μM). (E) Cell viability and (F) LDH release in HC11 cells pretreated with different concentrations of quercetin (0–25 μM) for 2 h, followed by treatment of 100 μM H_2O_2 for 24 h. One-way ANOVA followed by Tukey's multiple comparison was used to determine the differences between the groups. Data represent the mean ± SEM. Data marked with different small letters (a, b, c and d) indicated $P < 0.05$, while data with the same letter indicated no significant difference.

https://doi.org/10.1017/anr.2024.2 Published online by Cambridge University Press

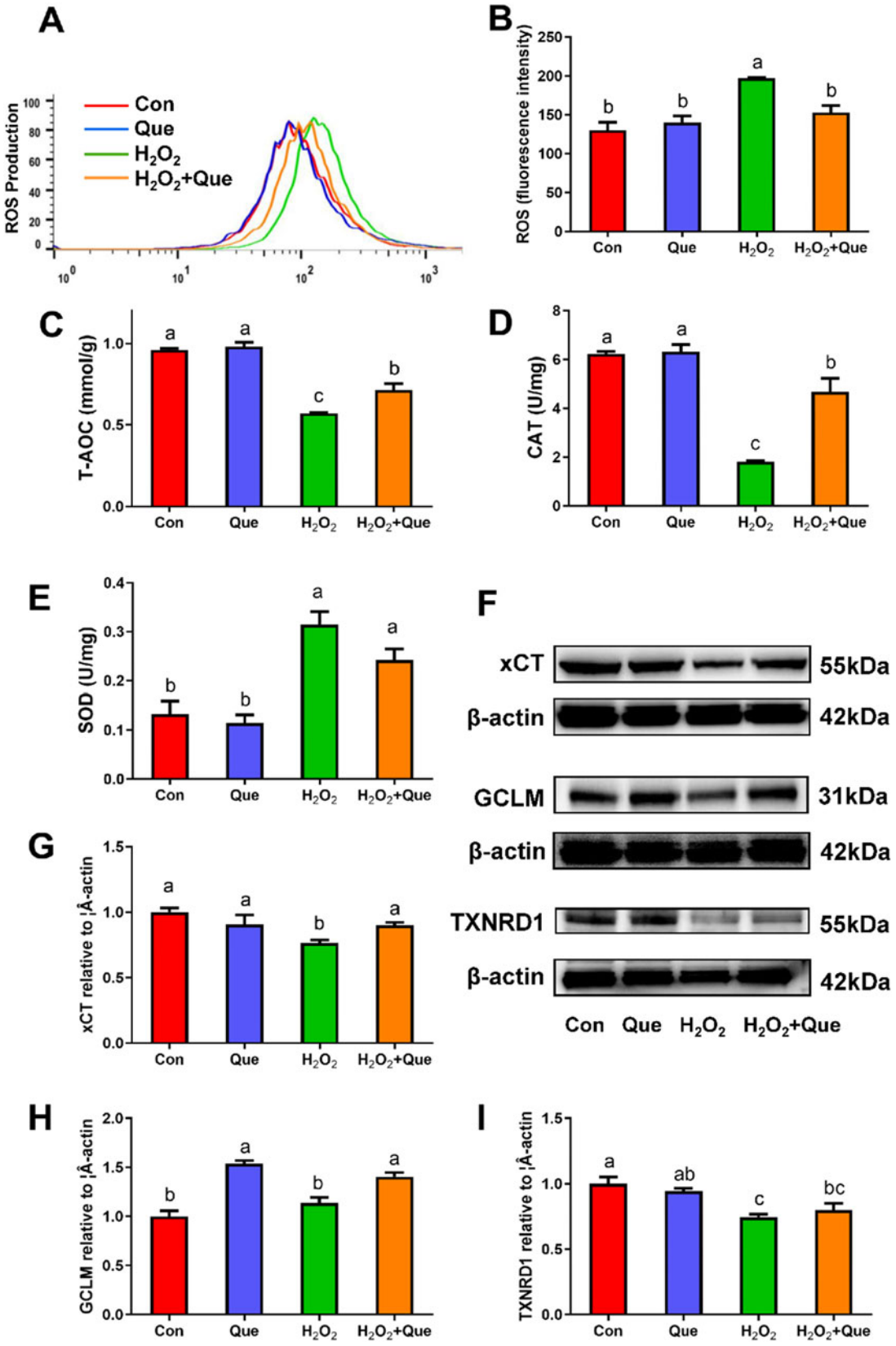

Figure 2. The ameliorating effects of quercetin on redox balance in HC11 cells. HC11 cells were pretreated with or without 20 μM quercetin for 2 h, followed by treatment with or without 100 μM H_2O_2 for 24 h to determine (A and B) the production of ROS, (C) T-AOC, enzyme activity of (D) CAT and (E) SOD. (F) Representative grayscale photographs of immunoblots and quantitative protein expression of (G) xCT, (H) GCLM and (I) TXNRD1. One-way ANOVA followed by Tukey's multiple comparison was used to determine the differences between four groups. Data represent mean ± SEM. Data marked with different lowercase letters (a, b and c) indicated $P < 0.05$, while data with the same letter indicated no significant difference.

https://doi.org/10.1017/anr.2024.2 Published online by Cambridge University Press

the cell viability (Fig. 1C). In addition, 5–25 μM quercetin did not affect the release of LDH from the cells (Fig. 1D). The LDH release induced by H_2O_2 was significantly reduced by 5–25 μM quercetin, and cell viability decreased by H_2O_2 was improved by 10–25 μM quercetin (Fig. 1E–F). As a result, 20 μM was the most effective concentration of quercetin in alleviating HC11 cell damage caused by H_2O_2.

Effect of quercetin on H_2O_2-induced redox imbalance

The production of ROS in HC11 cells increased by 100 μM H_2O_2 treatment, and quercetin pretreatment restored that to the control level (Fig. 2A–B). H_2O_2 treatment reduced the T-AOC in HC11 cells, which was significantly improved by quercetin pretreatment (Fig. 2C). H_2O_2 treatment significantly reduced the activity of CAT and increased the activity of SOD in the cells (Fig. 2D–E), and quercetin pretreatment significantly improved the activity of CAT reduced by H_2O_2 (Fig. 2D). H_2O_2 significantly inhibited the expression of glutamate/cystine reverse transporter light chain (xCT) and thioredoxin reductase 1 (TXNRD1), and the inhibition on the expression of xCT was completely removed by quercetin pretreatment (Fig. 2F–G, I). Quercetin partially restored the expression of TXNRD1 under H_2O_2 (Fig. 2F, I). Although H_2O_2 treatment did not affect the expression of glutamate-cysteine ligase modifier subunit (GCLM), quercetin treatment with or without H_2O_2 upregulated the expression of GCLM compared with the control group (Fig. 2F, H).

Effect of quercetin on the activation of MAPK pathway induced by H_2O_2

Hydrogen peroxide increased the level of phosphorylation of p38 MAPK and the ratio of p-p38 MAPK/p38 MAPK, and quercetin pretreatment restored the phosphorylation level of p38 MAPK and the ratio of p-p38 MAPK/p38 MAPK to the control levels (Fig. 3A–D). Similarly, H_2O_2 increased the phosphorylation level of ERK protein and the ratio of p-ERK/ERK and decreased the total protein level of ERK (Fig. 3A, E–G). Quercetin pretreatment restored the phosphorylation level of ERK, the ratio of p-ERK/ERK and the protein expression of ERK to a great extent (Fig. 3A, E–G). Meanwhile, H_2O_2 increased the ratio of p-JNK/JNK and decreased

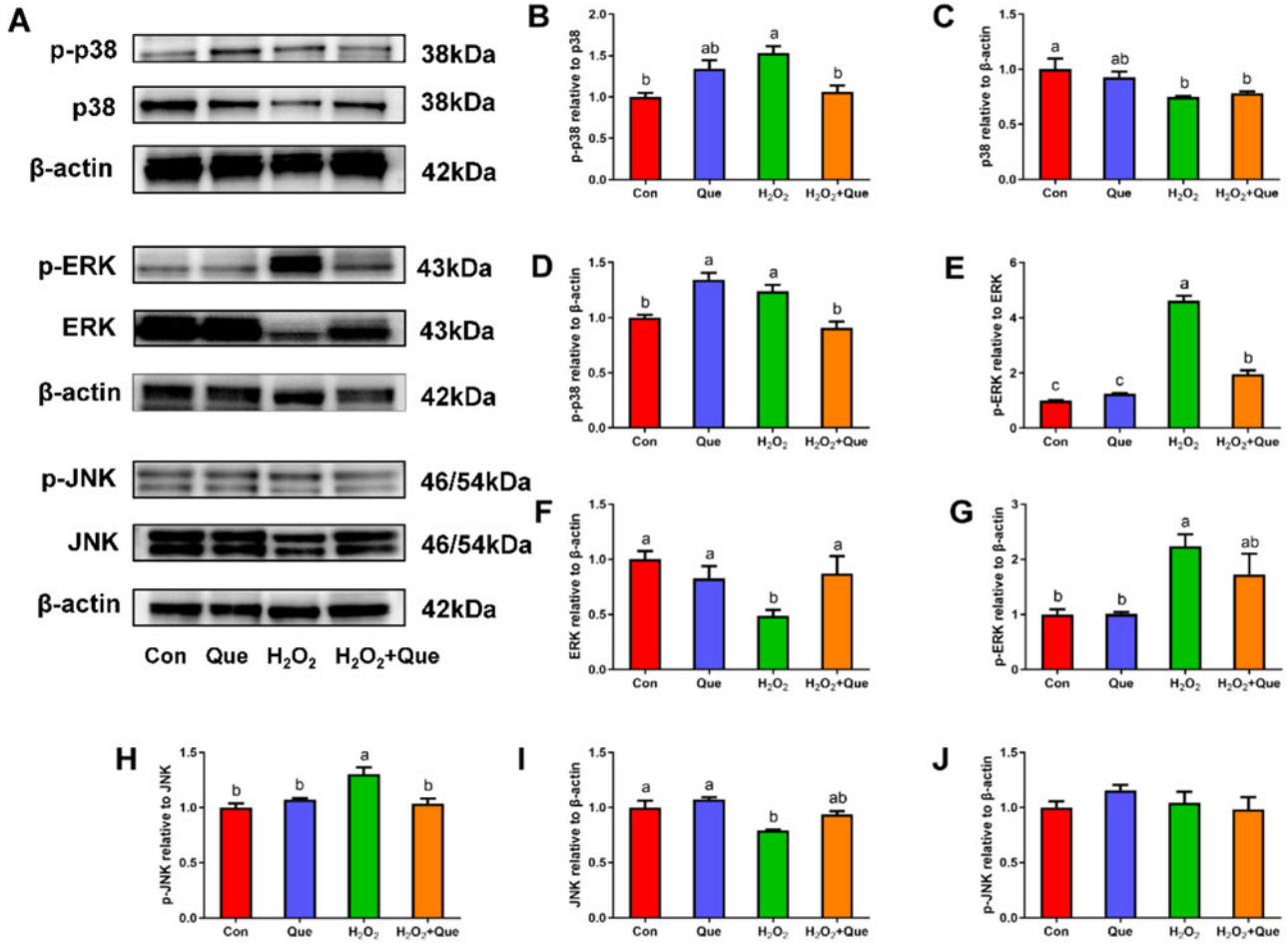

Figure 3. Effects of quercetin on the H_2O_2-induced activation of MAPK pathway in HC11 cells. HC11 cells were pretreated with or without 20 μM quercetin for 2 h, followed by treatment with or without 100 μM H_2O_2 for 24 h. (A) Representative grayscale photographs of immunoblots. Quantitative protein expression of (B) p-p38/p38, (C) p38, (D) p-p38, (E) p-ERK/ERK, (F) ERK, (G) p-ERK, (H) p-JNK/JNK, (I) JNK and (J) p-JNK. One-way ANOVA followed by Tukey's multiple comparison was used to determine the differences between four groups. Data represent the mean ± SEM. Data marked with different lowercase letters (a, b and c) indicated $P < 0.05$, while data with the same letter indicated no significant difference.

https://doi.org/10.1017/anr.2024.2 Published online by Cambridge University Press

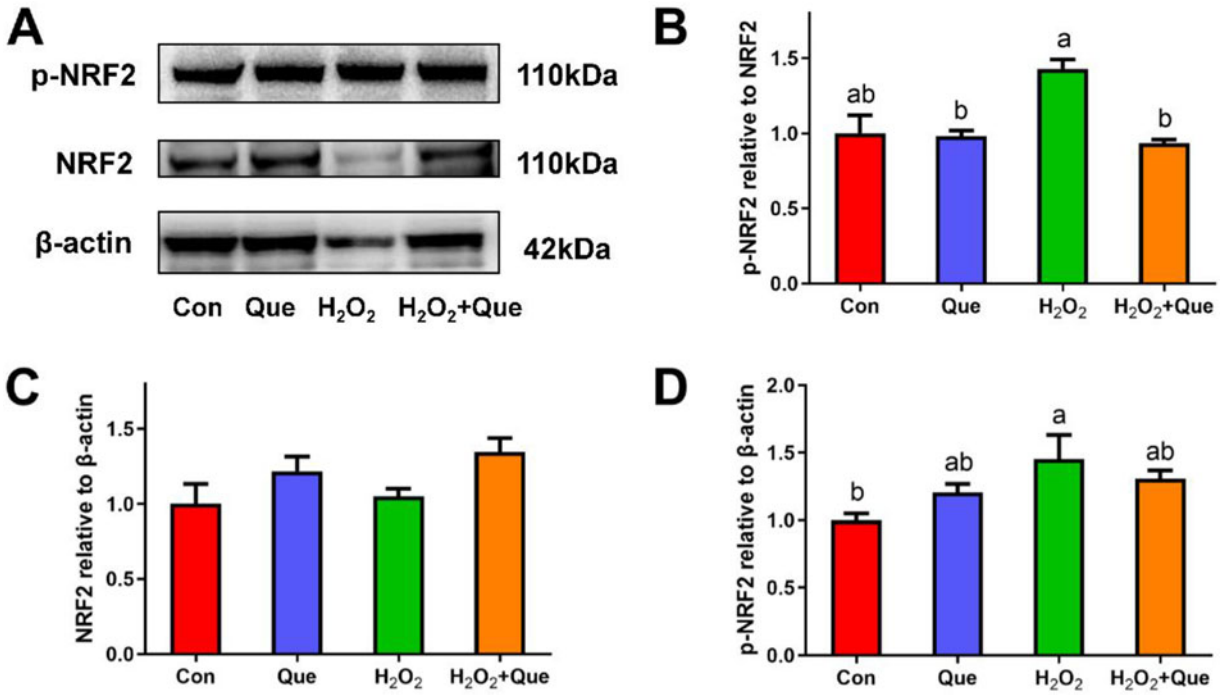

Figure 4. Effects of quercetin on the H_2O_2-induced activation of Nrf2 pathway in HC11 cells. HC11 cells were pretreated with or without 20 μM quercetin for 2 h, followed by treatment with or without 100 μM H_2O_2 for 24 h. (A) Representative grayscale photographs of immunoblots. (B) Quantitative presentation of the ratio of p-NRF2/NRF2 protein and protein expression level of (C) NRF2 and (D) p-NRF2. One-way ANOVA followed by Tukey's multiple comparison was used to determine the differences between four groups. Data represent mean ± SEM. Data marked with different lowercase letters (a and b) indicated $P < 0.05$, while data with the same letter indicated no significant difference.

the total protein level of JNK but did not affect the phosphorylation level of JNK, and quercetin pretreatment upregulated the level of JNK and downregulated the ratio of p-JNK/JNK to the control level in H_2O_2 treated cells (Fig. 3A, H–J).

Effect of quercetin on the activation of Nrf2 pathway induced by H_2O_2

Hydrogen peroxide significantly increased the protein expression of p-NRF2 and the ratio of p-NRF2/NRF2 but did not affect the total protein expression of NRF2 (Fig. 4A–D). Quercetin pretreatment restored the expression of p-NRF2 to the level in control cells, and the ratio of p-NRF2/NRF2 in quercetin pretreatment group was significantly lower than that in H_2O_2 group (Fig. 4).

Role of MAPK pathway in ameliorating H_2O_2-induced activation of Nrf2 pathway by quercetin

The p38 MAPK inhibitor (SB203580, 25 μM), ERK inhibitor (PD98059, 100 μM), JNK inhibitor (SP600125, 10 μM) and Nrf2 inhibitor (ML385, 5 μM) inhibited the activation of MAPK and Nrf2 pathways, respectively (Figure S1). The ERK inhibitor PD98059 significantly upregulated the ratio of p-NRF2/NRF2 and completely eliminated the recovery effect of quercetin on H_2O_2-induced activation of Nrf2 pathway in HC11 cells (Fig. 5). The p38 MAPK inhibitor SB203580 significantly reduced the protein expression of p-NRF2, whereas the JNK inhibitor SP600125 did not affect Nrf2 signaling pathway significantly in cells treated with H_2O_2 (Fig. 5).

Roles of MAPK and Nrf2 pathways in quercetin's ameliorating effect on H_2O_2-induced oxidative stress

All p38 MAPK, ERK and JNK inhibitors significantly reduced the protective effect of quercetin on cell viability (Fig. 6A). There were no differences in cell viability between any group of Nrf2, p38 MAPK and ERK inhibitors and the H_2O_2 group (Fig. 6A). No inhibitors significantly affected quercetin's alleviation in the release of LDH induced by H_2O_2 (Fig. 6B). Nrf2 inhibitor, but not MAPK pathway inhibitors, blocked the protective effect of quercetin on the level of ROS to a certain degree (Fig. 6C–D). In addition, the p38 MAPK, ERK and Nrf2 inhibitors decreased the recovery effect of quercetin on T-AOC of HC11 cells, and the inhibition of ERK pathway reduced the T-AOC to the level of the control group (Fig. 6E). Four inhibitors decreased quercetin's upregulation of CAT activity to different degrees (Fig. 6F). Nrf2 inhibitor, but not MAPK pathway inhibitors, significantly reduced the upregulation of quercetin on the protein level of xCT to the basal level of H_2O_2 group (Fig. 6G–H). Furthermore, the Nrf2 inhibitor eliminated the upregulation of quercetin on the expression of GCLM and restored it to the level of H_2O_2 group, whereas the expression of GCLM in three MAPK pathway inhibitor groups was between the H_2O_2 group and quercetin group. There was no difference in protein level of GCLM between the Nrf2 inhibitor and p38 MAPK inhibitor groups (Fig. 6G, I).

Discussion

Oxidative stress impacts mammary health seriously, resulting in decreased milk yield and milk quality (Li et al. 2019; Spitzer et al. 2020). Alleviating oxidative stress by using exogenous substances

https://doi.org/10.1017/anr.2024.2 Published online by Cambridge University Press

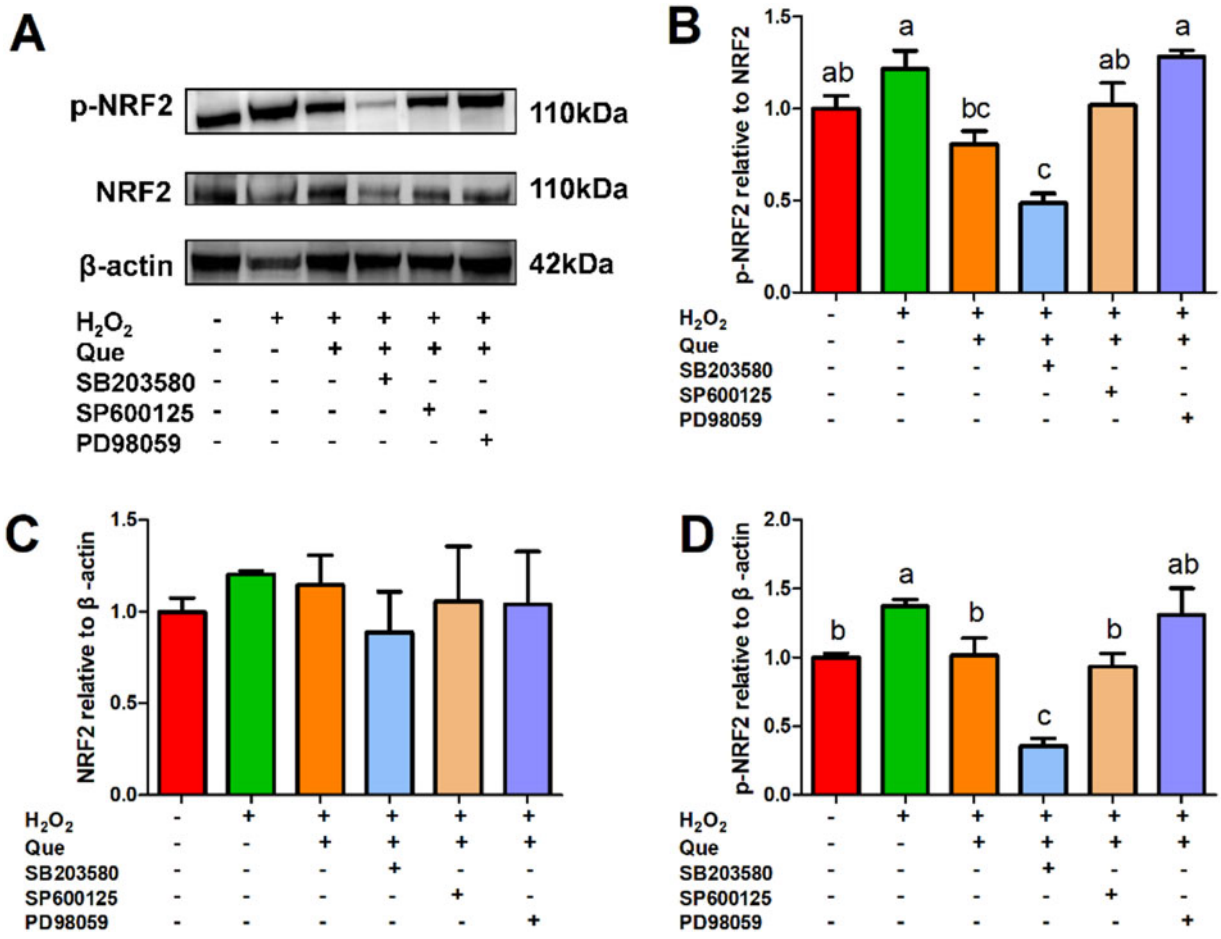

Figure 5. Effects of the p38 MAPK inhibitor SB203580 (25 μM), ERK inhibitor PD98059 (100 μM) and JNK inhibitor SP600125 (10 μM) on the quercetin's ameliorating effect on H_2O_2-induced activation of Nrf2 pathway in HC11 cells. HC11 cells were pretreated with or without an individual inhibitor for 1 h, then the cells were treated with or without 20 μM quercetin for 2 h, followed by treatment with or without 100 μM H_2O_2 for 24 h. (A) Representative grayscale photographs of immunoblots. (B) Quantitative presentation of the ratio of p-NRF2/NRF2 protein and the protein expression level of (C) NRF2 and (D) p-NRF2. One-way ANOVA followed by Tukey's multiple comparison was used to determine the differences between the groups. Data represent mean ± SEM. Data marked with different lowercase letters (a, b and c) indicated $P < 0.05$, while data with the same letter indicated no significant difference.

has been the focus of antioxidation research in the mammary gland (Koch et al. 2019; Xu et al. 2019). Mammary epithelial cells are lactigenous cells and directly related to the health and function of the mammary gland (Mizusawa et al. 2019; Yuan et al. 2019). Therefore, a model of cellular oxidative stress was adapted in HC11 mouse mammary epithelial cells with H_2O_2 treatment. In this model, the cell viability was gradually decreased by increasing H_2O_2 concentration from 50 μM to 250 μM. The 100 μM H_2O_2 reduced cell viability to about 60% of control group and significantly increased the release of LDH from the cells, which was then used for subsequent experiments. Quercetin is a flavonoid extracted from a variety of plants (Polera et al. 2019). Quercetin has shown to have antioxidant properties in previous studies (Song et al. 2020; Xu et al. 2019). In our study, HC11 cells were treated with quercetin at commonly used concentrations (Li et al. 2016; Smith et al. 2020) and found that quercetin below 25 μM had no negative effect on cell viability and LDH release from the cells. However, 5–20 μM quercetin had significant protective effects on the viability of HC11 cells treated with H_2O_2, and 20 μM quercetin showed the maximal effects, which was then used in subsequent experiments to study the underlying mechanisms.

ROS include reductive oxygen-containing free radicals and non-free radical reactive oxidants (Sessa et al. 2020). In this study, H_2O_2 treatment increased ROS production and decreased the T-AOC in HC11 cells, indicating that H_2O_2 resulted in redox imbalance, which was consistent with previous reports (Jin et al. 2016). There are numbers of antioxidant enzymes and non-enzymatic antioxidants in the body, such as CAT, SOD and xCT (Vucetic et al. 2017), which constitute the antioxidant defense system. Quercetin increased T-AOC and the activity of CAT reduced by H_2O_2, verifying that quercetin restored H_2O_2-induced redox imbalance in HC11 cells. The xCT is a member of the cystine/glutamate antiporter family and promotes cystine uptakes and protects cells from oxidative stress and cell death (Koppula et al. 2018). In this study, quercetin completely eliminated the reduction of xCT induced by H_2O_2 and partially restored the level of stress-resistant protein TXNRD1 in HC11 cells. These effects of quercetin on stress-resistant proteins were consistent with previous *in vivo* studies in pancreatic tissue of type 2 diabetes mice (Li et al. 2020b), proving quercetin's antioxidation function *in vivo* and *in vitro*. Moreover, GCLM is the regulatory subunit of GCL which affects the synthesis of glutathione (GSH) (Chen et al. 2019; Nishizawa et al. 2020). The mRNA abundance of GCLM was found to be

https://doi.org/10.1017/anr.2024.2 Published online by Cambridge University Press

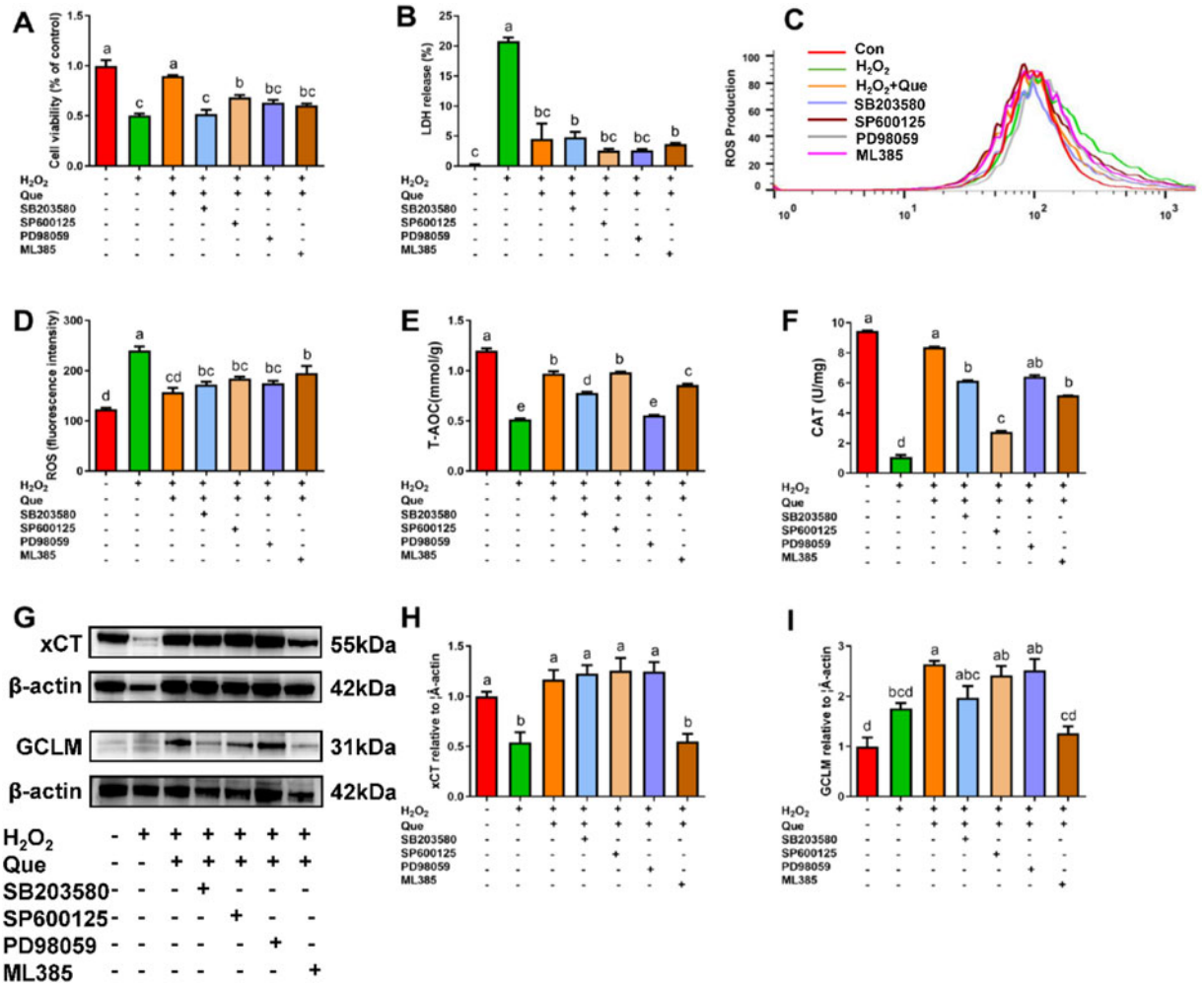

Figure 6. Effects of the p38 MAPK inhibitor SB203580 (25 μM), ERK inhibitor PD98059 (100 μM), JNK inhibitor SP600125 (10 μM) and Nrf2 inhibitor ML385 (5 μM) on the quercetin's ameliorating effects on H_2O_2-induced oxidative stress in HC11 cells. HC11 cells were pretreated with or without an individual inhibitor for 1 h, then the cells were treated with or without 20 μM quercetin for 2 h, followed by treatment with or without 100 μM H_2O_2 for 24 h to determine (A) cell proliferation, (B) LDH release, (C and D) the production of ROS, (E) T-AOC and (F) CAT enzyme activity. (G) Representative grayscale photographs of immunoblots. Quantitative protein expression of (H) xCT and (I) GCLM. One-way ANOVA followed by Tukey's multiple comparison was used to determine the differences between the groups. Data represent mean ± SEM. Data marked with different lowercase letters (a, b, c, d and e) indicated $P < 0.05$, while data with the same letter indicated no significant difference.

downregulated after H_2O_2 treatment in human retinal pigment epithelial cells (ARPE-19 cells) (Yuan et al. 2020). In the present study, the protein level of GCLM was not changed by H_2O_2, but it was induced by quercetin treatment, indicating that GCLM may still be one of the important molecules involved in quercetin protection in HC11 cells.

The MAPK signaling pathway is a key pathway that regulates cell proliferation and respond to exogenous stimuli, and MAPK signaling molecules mainly include p38 MAPK, ERK and JNK subtypes (Lanna et al. 2017; Santarpia et al. 2012). Studies on cardiac fibroblasts have shown that quercetin reduces the phosphorylation of ERK, p38 and JNK induced by free radicals, but there have only a few studies which investigated the relationship between quercetin and MAPK pathway in mammary epithelial cells (Min et al. 2019). In this study, H_2O_2 activated the p38 MAPK, ERK and JNK molecules by phosphorylation, and quercetin restored the activation of these molecules, revealing that MAPK pathway might play a role in the protective effects of quercetin in mammary epithelial cells. Similarly, quercetin decreased p-NRF2/NRF2 ratio in cells treated with H_2O_2, proving that Nrf2 pathway may also likely to be involved in quercetin's protective role to oxidative stress. These results are consistent with the reports that quercetin is a Nrf2-interacting nutrient in improving Alzheimer's disease, insulin resistance and lung injury (Bousquet et al. 2020; Zaplatic et al. 2019).

To investigate the molecular mechanisms of quercetin protection against oxidative stress in HC11 cells, the specific inhibitors of Nrf2 (ML385), p38 MAPK (SB203580), ERK (PD98059) and JNK (SP600125) molecule (Jin et al. 2016; Martin-Acosta and Xiao 2021; Zarrin et al. 2021) were used to explore the role of each of these pathways in quercetin's function. The inhibition of ERK upregulated the ratio of p-NRF2/NRF2 and eliminated the recovery effect of quercetin on Nrf2 signaling pathway, whereas p38 MAPK inhibition reduced the protein level of p-NRF2, indicating that quercetin may protect HC11 cells by activating ERK-Nrf2 and p38 MAPK-Nrf2 pathways simultaneously. The relationship between MAPK and Nrf2 signaling pathways has not been consistent in different studies (Jin et al. 2016; Li et al. 2020a; Liu et al. 2020a). For example, ERK inhibition decreased, whereas p38 MAPK inhibition increased the activation state of Nrf2 signaling

https://doi.org/10.1017/anr.2024.2 Published online by Cambridge University Press

pathway under resveratrol treatment (Jin et al. 2016). All three MAPK pathway inhibitors activated the expression of Nrf2 in particulate matter $(PM)_{2.5}$-induced spermatogenesis dysfunction (Liu et al. 2020a). Thus, the roles of MAPK and Nrf2 signaling pathways may be cell-specific or rely on the biological activity of different antioxidants.

Yao et al. (2007) found that the inhibitors of p38 MAPK and ERK blocked the protective effects of quercetin on alcoholic liver injury, whereas JNK inhibitor failed to do that. In this study, the Nrf2, p38 MAPK and ERK inhibitors completely blocked the effect of quercetin on cell proliferation, but the JNK inhibitor only blocked partially. Our observation was similar to the result of Yao et al. (2007) in alcoholic liver injury, suggesting that the antioxidation mechanisms of quercetin might be at least partially determined by the structure and characteristics of quercetin itself. Besides, the level of ROS is an indicator of cellular oxidation stress (Bottje 2019). Previous reports showed that p38 MAPK inhibitor reduced the elevation of ROS induced by ochratoxin A, and Nrf2 knockdown upregulated ROS level in rat hippocampal neurons, but the effects of Nrf2 and MAPK inhibitors on ROS of HC11 cells were not clear (Dai et al. 2018; Han et al. 2019). In this study, Nrf2 inhibitor, but not MAPK inhibitors, partially blocked the down-regulation effect of quercetin on ROS production. However, due to the presence of ERK-Nrf2 and p38 MAPK-Nrf2 pathways, MAPK pathway may also indirectly affect ROS production at other time points. Moreover, our observations indicated that p38 MAPK, ERK and Nrf2 pathways are all necessary in the enhancement of T-AOC by quercetin in HC11 cells, whereas JNK pathway is not. But interestingly, the JNK inhibitor had the greatest effect on CAT activity among four inhibitors. The changes of T-AOC and CAT activity were not completely consistent after 24 h of H_2O_2 treatment, suggesting that T-AOC in HC11 cells was determined by many antioxidant substances other than CAT enzyme, as other reports in the liver (Jia et al. 2019; Liu et al. 2020b).

Several studies have confirmed that xCT is a downstream gene of the Nrf2-antioxidant response element pathway (Xie et al. 2020; Xie et al. 2020). Repression of the activation of Nrf2-antioxidant response element pathway by si-RNA or overexpression of Nrf2's anchor protein KEAP1 inhibited the expression of xCT (Ali et al. 2016; Habib et al. 2015). In this study, the Nrf2 inhibitor completely blocked H_2O_2-induced protein expression of xCT, which was consistent with other studies *in vivo* and *in vitro* (Ali et al. 2016; Xie et al. 2020). However, three MAPK inhibitors did not affect the expression of xCT. Hence, the effect of quercetin on xCT in HC11 cells was mainly dependent on the Nrf2 pathway. Ishikado *et al.* found that different si-RNAs of Nrf2 inhibited the expression of GCLM in liver and vascular endothelial cells, and the inhibitor or si-RNA of p38 MAPK also had the inhibitory effects on GCLM (Gu et al. 2016; Ishikado et al. 2013; Ji et al. 2015). In HC11 cells, the recovery effect of quercetin on the protein level of GCLM was largely dependent on Nrf2 and p38 MAPK pathways and also partially relied on JNK and ERK pathways, proving that the level of GCLM may be affected by two pathways simultaneously.

Conclusion

In summary, our study showed that quercetin protected HC11 cells against H_2O_2-induced redox imbalance by regulating cell viability, ROS production, enzyme activity of CAT and the expression of stress-resistant proteins via MAPK and Nrf2 signaling pathways.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/anr.2024.2.

Author contributions. Yongxin Li: conceptualization, investigation, visualization and writing – original draft. **Ning Han**: methodology and investigation. Pengfei Hou: investigation. Feng-Qi Zhao: writing – review and editing. Hongyun Liu: funding acquisition, writing – review and editing, and supervision.

Financial support. This research was supported by grants from the National Natural Science Foundations of China (32072756) and the China Agricultural Research System (Grant No. CARS-36). The funders had no role in the design, analysis or writing of this article.

Conflicts of interest. The authors declare no conflicts of interest.

References

Ali D, Mohammad DK, Mujahed H, Jonson-Videsäter K, Nore B, Paul C and Lehmann S (2016) Anti-leukaemic effects induced by APR-246 are dependent on induction of oxidative stress and the NFE2L2/HMOX1 axis that can be targeted by PI3K and mTOR inhibitors in acute myeloid leukaemia cells. *British Journal of Haematology* **174**, 117–126.

Bottje WG (2019) Oxidative metabolism and efficiency: The delicate balancing act of mitochondria. *Poultry Science* **98**, 4223–4230.

Bousquet J, Cristol JP, Czarlewski W, Czarlewski W, Anto JM, Martineau A, Haahtela T, Fonseca SC, Laccarino G, Blain H, Fiocchi A, Canonica GW, Fonseca JA, Vidal A, Choi HJ, Kim HJ, Le Moing V, Reynes J, Sheikh A, Akdis CA and Zuberbier T (2020) Nrf2-interacting nutrients and COVID-19: Time for research to develop adaptation strategies. *Clinical &Translational Allergy* **10**, 58.

Chen X, Xue H, Fang W, Chen K, Chen S, Yang W, Shen T, Chen X, Zhang P and Ling W (2019) Adropin protects against liver injury in nonalcoholic steatohepatitis via the Nrf2 mediated antioxidant capacity. *Redox Biology* **21**, 101068.

Chen Q, Zhao FQ, Ren Y, Han J, Liu J, Li Y and Liu H (2020) Parenterally delivered methionyl-methionine dipeptide during pregnancy enhances mammogenesis and lactation performance over free methionine by activating PI3K-AKT signaling in methionine-deficient mice. *The Journal of Nutrition* **150**, 1186–1195.

Dai Y, Zhang H, Zhang J and Yan M (2018) Isoquercetin attenuates oxidative stress and neuronal apoptosis after ischemia/reperfusion injury via Nrf2-mediated inhibition of the NOX4/ROS/NF-κB pathway. *Chemico-Biological Interactions* **284**, 32–40.

Garwolinska D, Namiesnik J, Kot-Wasik A and Hewelt-Belka W (2018) Chemistry of human breast milk—A comprehensive review of the composition and role of milk metabolites in child development. *Journal of Agricultural and Food Chemistry* **66**, 11881–11896.

Gu L, Tao X, Xu Y, Han X, Qi Y, Xu L, Yin L and Peng J (2016) Dioscin alleviates BDL- and DMN-induced hepatic fibrosis via Sirt1/Nrf2-mediated inhibition of p38 MAPK pathway. *Toxicology and Applied Pharmacology* **292**, 19–29.

Habib E, Linher-Melville K, Lin HX and Singh G (2015) Expression of xCT and activity of system xc(-) are regulated by NRF2 in human breast cancer cells in response to oxidative stress. *Redox Biology* **5**, 33–42.

Han Z, Zhang Y, Wang C, Liu X, Jiang A, Liu Z, Wang J, Yang Z and Wei Z (2019) Ochratoxin A-triggered chicken heterophil extracellular traps release through reactive oxygen species production dependent on activation of NADPH oxidase, ERK, and p38 MAPK signaling pathways. *Journal of Agricultural and Food Chemistry* **67**, 11230–11235.

Ishikado A, Sono Y, Matsumoto M, Robida-Stubbs S, Okuno A, Goto M, King GL, Keith Blackwell T and Makino T (2013) Willow bark extract increases antioxidant enzymes and reduces oxidative stress through activation of Nrf2 in vascular endothelial cells and Caenorhabditis elegans. *Free Radical Biology and Medicine* **65**, 1506–1515.

Jakubczyk K, Dec K, Kaldunska J, Kawczuga D, Kochman J and Janda K (2020) Reactive oxygen species – sources, functions, oxidative damage. *Polski Merkuriusz Lekarski* **48**, 124–127.

Jia YN, Peng YL, Zhao YP, Cheng XF, Zhou Y, Chai CL, Zeng LS, Pan MH and Xu L (2019) Comparison of the hepatoprotective effects of the three main stilbenes from mulberry twigs. *Journal of Agricultural and Food Chemistry* **67**, 5521–5529.

Jin X, Wang K, Liu H, Hu F, Zhao F and Liu J (2016) Protection of bovine mammary epithelial cells from hydrogen peroxide-induced oxidative cell damage by resveratrol. *Oxidative Medicine and Cellular Longevity* **2016**, 2572175.

Ji LL, Sheng YC, Zheng ZY, Shi L and Wang ZT (2015) The involvement of p62-Keap1-Nrf2 antioxidative signaling pathway and JNK in the protection of natural flavonoid quercetin against hepatotoxicity. *Free Radical Biology and Medicine* **85**, 12–23.

Koch W, Zagorska J, Marzec Z and Kukula-Koch W (2019) Applications of tea (Camellia sinensis) and its active constituents in cosmetics. *Molecules* **24**, 4277.

Koppula P, Zhang Y, Zhuang L and Gan B (2018) Amino acid transporter SLC7A11/xCT at the crossroads of regulating redox homeostasis and nutrient dependency of cancer. *Cancer Communications* **38**, 12.

Kumar S, Singh SK, Rana B and Rana A (2021) The regulatory function of mixed lineage kinase 3 in tumor and host immunity. *Pharmacology & Therapeutics* **219**, 107704.

Lanna A, Gomes DC, Muller-Durovic B, McDonnell T, Escors D, Gilroy DW, Lee JH, Karin M and Akbar AN (2017) A sestrin-dependent Erk-Jnk-p38 MAPK activation complex inhibits immunity during aging. *Nature Immunology* **18**, 354–363.

Li D, Jiang C, Mei G, Zhao Y, Chen L, Liu J, Tang Y, Gao C and Yao P (2020b) Quercetin alleviates ferroptosis of pancreatic beta cells in type 2 diabetes. *Nutrients* **12**, 2954.

Li XS, Tang XY, Su W and Li X (2020a) Vitexin protects melanocytes from oxidative stress via activating MAPK-Nrf2/ARE pathway. *Immunopharmacology and Immunotoxicology* **42**, 594–603.

Liu S, Chang X, Yu J and Xu W (2020b) Cerasus humilis cherry polyphenol reduces high-fat diet-induced obesity in C57BL/6 mice by mitigating fat deposition, inflammation, and oxidation. *Journal of Agricultural and Food Chemistry* **68**, 4424–4436.

Liu B, Shen LJ, Zhao TX, Sun M, Wang JK, Long CL, He DW, Lin T, Wu SD and Wei GH (2020a) Automobile exhaust-derived $PM_{2.5}$ induces blood-testis barrier damage through ROS-MAPK-Nrf2 pathway in sertoli cells of rats. *Ecotoxicology & Environmental Safety* **189**, 110053.

Li C, Wang Y, Li L, Han Z, Mao S and Wang G (2019) Betaine protects against heat exposure-induced oxidative stress and apoptosis in bovine mammary epithelial cells via regulation of ROS production. *Cell Stress and Chaperones* **24**, 453–460.

Li C, Zhang WJ and Frei B (2016) Quercetin inhibits LPS-induced adhesion molecule expression and oxidant production in human aortic endothelial cells by p38-mediated Nrf2 activation and antioxidant enzyme induction. *Redox Biology* **9**, 104–113.

Luo Z, Xu X, Sho T, Zhang J, Xu W, Yao Y and Xu J (2019) ROS-induced autophagy regulates porcine trophectoderm cell apoptosis, proliferation, and differentiation. *American Journal of Physiology - Cell Physiology* **316**, 198–209.

Martin-Acosta P and Xiao X (2021) PROTACs to address the challenges facing small molecule inhibitors. *European Journal of Medicinal Chemistry* **210**, 112993.

Min Z, Yangchun L, Yuquan W and Changying Z (2019) Quercetin inhibition of myocardial fibrosis through regulating MAPK signaling pathway via ROS. *Pakistan Journal of Pharmaceutical Sciences* **32**, 1355–1359.

Mizusawa M, Sharmin MM and Yonekura S (2019) Mild heat stress induces transcription of the beta-casein gene via unfolded protein response-activated XBP1 signaling in undifferentiated mammary epithelial cells. *Animal Science Journal* **90**, 1026–1032.

Nishizawa H, Matsumoto M, Shindo T, Saigusa DD, Kato K, Suzuki K, Sato M, Ishii Y, Shimokawa H and Igarashi K (2020) Ferroptosis is controlled by the coordinated transcriptional regulation of glutathione and labile iron metabolism by the transcription factor BACH1. *Journal of Biological Chemistry* **295**, 69–82.

Polera N, Badolato M, Perri F, Carullo G and Aiello F (2019) Quercetin and its natural sources in wound healing management. *Current Medicinal Chemistry* **26**, 5825–5848.

Santarpia L, Lippman SM and El-Naggar AK (2012) Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. *Expert Opinion on Therapeutic Targets* **16**, 103–119.

Sessa F, Messina G, Russo R, Salerno M, Castruccio C, Distefano A, Li Volti G, Calogero AE, Cannarella R, Mongioi LM, Condorelli RA and La Vignera S (2020) Consequences on aging process and human wellness of generation of nitrogen and oxygen species during strenuous exercise. *The Aging Male* **23**, 14–22.

Shaw P and Chattopadhyay A (2020) Nrf2-ARE signaling in cellular protection: Mechanism of action and the regulatory mechanisms. *Journal of Cellular Physiology* **235**, 3119–3130.

Smith MJ, Fowler M, Naftalin RJ and Siow RCM (2020) UVA irradiation increases ferrous iron release from human skin fibroblast and endothelial cell ferritin: Consequences for cell senescence and aging. *Free Radical Biology and Medicine* **155**, 49–57.

Song X, Wang Y and Gao L (2020) Mechanism of antioxidant properties of quercetin and quercetin-DNA complex. *Journal of Molecular Modeling* **26**, 133.

Spitzer AJ, Tian Q, Choudhary RK and Zhao FQ (2020) Bacterial endotoxin induces oxidative stress and reduces milk protein expression and hypoxia in the mouse mammary gland. *Oxidative Medicine and Cellular Longevity* **2020**, 3894309.

Tian R, Yang Z, Lu N and Peng YY (2019) Quercetin, but not rutin, attenuated hydrogen peroxide-induced cell damage via heme oxygenase-1 induction in endothelial cells. *Archives of Biochemistry and Biophysics* **676**, 108157.

Vucetic M, Cormerais Y, Parks SK and Pouysségur J (2017) The central role of amino acids in cancer redox homeostasis: Vulnerability points of the cancer redox code. *Frontiers in Oncology* **7**, 319.

Xie LW, Cai S, Zhao TS, Li M and Tian Y (2020) Green tea derivative (-)-epigallocatechin-3-gallate (EGCG) confers protection against ionizing radiation-induced intestinal epithelial cell death both in vitro and in vivo. *Free Radical Biology and Medicine* **161**, 175–186.

Xu D, Hu MJ, Wang YQ and Cui YL (2019) Antioxidant activities of quercetin and its complexes for medicinal application. *Molecules* **24**, 1123.

Xu Y, Ke H, Li Y, Xie L, Su H, Xie J, Mo J and Chen W (2021) Malvidin-3-O-glucoside from blueberry ameliorates nonalcoholic fatty liver disease by regulating transcription factor EB-mediated lysosomal function and activating the Nrf2/ARE signaling pathway. *Journal of Agricultural and Food Chemistry* **69**, 4663–4673.

Yao P, Nussler A, Liu L, Hao L, Song F, Schirmeier A and Nussler N (2007) Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf2 pathways. *Journal of Hepatology* **47**, 253–261.

Yuan Z, Du W, He X, Zhang D and He W (2020) Tribulus terrestris ameliorates oxidative stress-induced ARPE-19 cell injury through the PI3K/Akt-Nrf2 signaling pathway. *Oxidative Medicine and Cellular Longevity* **2020**, 7962393.

Yuan X, Zhen Z, Zhang M, Yu Y, Gao X and Ao JX (2019) Cyclase-associated protein 1 is a key negative regulator of milk synthesis and proliferation of bovine mammary epithelial cells. *Cell Biochemistry & Function* **37**, 185–192.

Zaplatic E, Bule M, Shah S, Uddin M and Niaz K (2019) Molecular mechanisms underlying protective role of quercetin in attenuating Alzheimer's disease. *Life Science* **224**, 109–119.

Zarrin AA, Bao K, Lupardus P and Vucic D (2021) Kinase inhibition in autoimmunity and inflammation. *Nature Reviews Drug Discovery* **20**, 39–63.

https://doi.org/10.1017/anr.2024.2 Published online by Cambridge University Press