

Quercetin as a Lyn kinase inhibitor inhibits IgE-mediated allergic conjunctivitis

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ABSTRACT

Background: Allergic conjunctivitis (AC) resulting from conjunctival reactive inflammation is a common ocular surface disease. Quercetin is known for its anti-allergic properties but its effects on conjunctivitis are less well understood.

Purpose: In this study, we evaluated the anti-allergic effects of quercetin in animal models of conjunctivitis, and explored its molecular mechanism(s) of action in cultured human mast cells (MCs).

Key results: Quercetin inhibited the ovalbumin (OVA) induced expression of IgE, HA, IL-4, TNF- α and substance P in the peripheral blood of AC mouse models. Quercetin also attenuated OVA induced MC degranulation, eosinophil number, substance P concentrations, and mRNA IL-4/TNF- α expression in the conjunctival tissue of AC models. In vitro analysis showed that quercetin reduced DNP-HSA/IgE induced calcium (Ca^{2+}) influx, and suppressed degranulation and chemokine release in LAD2 cells (human primary mast cell). Quercetin also inhibited DNP-HSA/IgE induced Lyn/PLC γ /IP3R- Ca^{2+} activation, Lyn/ERK1/2 signaling, and Lyn/NF- κ B activation in LAD2 cells, all of which promote inflammation. When added alone, quercetin had no effect on PLC γ 1 phosphorylation or expression, but potently inhibited Lyn and phosphorylation-Lyn. Quercetin (200 μ M) and Lyn inhibitors (Bafetinib, 10 μ M) inhibit the activity of Lyn kinase, and quercetin can reduce the activation of Lyn kinase by Lyn agonist (Tolimidone, 10 μ M). These data can be preliminarily determined that quercetin can inhibit allergic conjunctivitis as a Lyn kinase inhibitor.

Conclusions and implications: This study illustrated the use of quercetin for the treatment of allergic conjunctivitis, which might act through its ability to inhibit Lyn/PLC γ /IP3R- Ca^{2+} , Lyn/ERK1/2, and Lyn/NF- κ B signaling. The inhibition of Lyn likely represents a major mechanism by which quercetin dampens the inflammatory response in AC disease models.

1. Introduction

Allergy is an acquired hypersensitivity reaction of the immune system in response to normally innocuous environmental substances (Cheng et al., 2013). The incidence of anaphylaxis (a severe allergic reaction) in western countries is in the range of 4–50 per 100,000 individuals per year (Lee and Vadas, 2011). Mast cells (MCs) are the primary effectors of allergic reactions and move to sites exposed to the external environment, including skin, the respiratory tract, and oral/gastrointestinal mucosa (Subramanian et al., 2016). MCs play an

important role in allergies through the secretion of histamines (HAs) and various inflammatory and immunomodulatory substances. In allergic pathologies, HA triggers acute symptoms due to its rapid effects on the vascular endothelium, bronchial cells, and smooth muscle. This leads to vasodilatation, vascular hyperpermeability, bronchoconstriction, hypotension, itching, cramping, diarrhea, and cutaneous wheal and flare responses (Su et al., 2014).

MCs are activated following IgE receptor (Fc ϵ RI) crosslinking, resulting in the release of a diverse array of preformed cytoplasmic granule-associated mediators, including HA and β -hexosaminidase, in

Abbreviations: AC, allergic conjunctivitis; MCs, human mast cells; OVA, ovalbumin; HA, histamines; LAD2, the laboratory of allergic disease 2 (a human primary mast cell); DNP-HAS, dinitrophenylated human serum albumin; Lyn, recombinant human tyrosine-protein kinase; PLC γ 1, phosphoinositide phospholipase γ 1; IP3R, inositol 1,4,5-trisphosphate receptor; IL-4, interleukin-4; TNF- α , tumor necrosis factor- α ; NF- κ B, nuclear factor-kappa B; ERK1/2, extracellular signal-regulated kinases 1/2; Oro, olopatadine

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addition to newly synthesized proinflammatory lipid mediators, cytokines and chemokines (Theoharides et al., 1982; Grimaldeston et al., 2005). IgE-Fcε RI-mediated MC signaling results in the activation of protein kinase C (PKC), and subsequent Ca^{2+} release from endoplasmic reticulum (ER) stores, which induces stromal interaction molecule 1-mediated opening of the store-operated Ca^{2+} channel ORAI1, leading to extracellular Ca^{2+} influx. Increased intracellular Ca^{2+} and the simultaneous activation of PKC triggers MC degranulation (Grimaldeston, 2015; Wernersson and Pejler, 2014; Beaven et al., 1984). Mitochondrial Ca^{2+} (Ca^{2+} [m]) uptake buffers the increase in local or bulk cytosolic Ca^{2+} (Ca^{2+} [c]) reduces its detrimental effects on MC viability (Pizzo et al., 2012).

Allergic conjunctivitis (AC) results in conjunctival reactive inflammation and is one of the most common ocular surface diseases (Origlieri and Bielory, 2009). AC manifests as itchy eyes, photophobia, eyelid edema, conjunctival hyperemia, and increased secretions. In severe cases, AC can lead to a loss of vision and corneal damage, severely influencing both learning ability and the quality of life of afflicted individuals (Liu et al., 1999). AC has a high incidence, extended disease progression, repeated attacks and limited therapeutic efficacy (Liu et al., 1999). Until now, the pathogenesis of AC is complex and its disease processes are poorly characterized. It is generally believed that AC is an immune disease mediated by MCs and IgE (Chigbu, 2009). Degranulation mediated MC factor release occurs in response to chemotactically active substances, including granulocyte chemokines, allergic eosinophil chemokines, and LT β 4, which attract inflammatory factors including neutrophils to the MC activation response sites. MC release also occurs in response to proinflammatory substances, including HA, PAF, tryptase and kininogenase, leading to vasodilation, edema, and tissue damage. Cariogenic substances including HA, prostaglandin D $_2$ (PGD $_2$), LTC $_4$ and LTD $_4$, which cause bronchial smooth muscle disease, also promote MC release. HA release causes a plethora of symptoms including itching, congestion, edema and tearing in the eye (Cook et al., 2001). It has been shown that activated MCs secrete inflammatory factors including interleukin 4 (IL-4) and tumor necrosis factor- α (TNF- α) which influence ocular surface immune regulation and aggravate AC symptoms (Leonardi, 2002). It has been shown that IL-4 and TNF- α promote and maintain allergic reactions. IL-4 increases the B-cell mediated production of IgE (Kuhn et al., 1991) and promotes T-cell differentiation into Th2 cells (Colleen, 2002). TNF- α promotes the chemotaxis of eosinophils, neutrophils, and giant cells, increasing cytotoxicity and the release of MC mediators (Colleen, 2002). The ability to stabilize MCs and prevent their degranulation, reduces HA and allergic mediator release, thereby preventing AC (Cook et al., 2002).

Until now, AC therapeutics can be classified into anti-histamines, MC stabilizers, non-steroidal anti-inflammatory drugs, hormones, and immunosuppressants. However, anti-histamines and MC stabilizers do not inhibit the release of neutrophils and eosinophils. The long-term use of glucocorticoids leads to side effects including drug-induced glaucoma, cataracts, and Cushing's syndrome. It is therefore of great clinical importance to identify drugs that can treat AC and rapidly control inflammation with minimal side-effects (Mishra et al., 2011).

Naturally occurring flavonoids such as quercetin, are polyphenolic compounds present in fruits, vegetables, nuts, seeds, herbs, spices and red wine, with anti-oxidant properties (Huxley et al., 2004; Schroeter et al., 2003). Flavonoids have potent anti-oxidant, anti-inflammatory (Middleton et al., 2000), and MC blocking activities (Kempuraj et al., 2005; Park et al., 2008) and can inhibit HA release from murine MCs (Foreman, 1984; Middleton, 1998), as well as IL-6 and TNF release from bone marrow-derived cultured murine MCs and rat peritoneal MCs (Kimata et al., 2000). Quercetin and the structurally related luteolin inhibit the release of HA, leukotrienes, and PGD $_2$ from human cultured MCs in response to the cross-linking of high affinity surface IgE receptors (FcεRI) (Kimata et al., 2000). Quercetin also inhibits HA, IL-6, IL-8, TNF- α and tryptase release from human MCs (Kempuraj et al.,

2005; Park et al., 2008) and prevents asthma development in animal models (Moon et al., 2008). Quercetin (10 mM) inhibits stimulated rat peritoneal MCs by ~75%, whilst 1 mM cromolyn was required for ~65% inhibition (Ennis et al., 1981). Quercetin is genotoxic. Oral dietary exposure at up to 1900 mg/kg/day for 15 months showed no treatment-related effects on survival and no treatment-related clinical signs of toxicity in (male and female F344/N rats). However, high doses of quercetin reduced body weight compared with controls during the last half of the study. Following two years of oral exposure, toxic and neoplastic lesions were seen in the kidney of male rats, including increased severity of chronic nephropathy, hyperplasia, and neoplasia of the renal tubular epithelium (Dunnick, 1992). A critical evaluation of the available literature on the biological effects of quercetin, including data related to its safety, concluded that quercetin, at estimated dietary intake levels (daily doses of supplemental quercetin of 200–1200 mg), would not produce adverse health effects (Harwood et al., 2007). However, quercetin has not been studied for its effects on allergic conjunctivitis in C57BL/6 mice, and its mechanisms of action in LAD2 cells remains uncharacterized.

In this study, we describe the effects of quercetin in the treatment of OVA-induced allergic conjunctivitis in C57BL/6 mice. We further reveal the ability of quercetin to inhibit DNP-HSA/IgE induced Ca^{2+} flux, MC degranulation, and chemokine release in LAD2 cells. Quercetin inhibited DNP-HSA/IgE induced Lyn/PLC γ /IP3R- Ca^{2+} signaling, Lyn/ERK1/2, and Lyn/NF- κ B signaling activation. Although quercetin did not influence PLC γ 1 levels or PLC γ 1 phosphorylation, it inhibited Lyn and phosphorylation-Lyn activation. Quercetin (200 μ M) and Lyn inhibitors (Bafetinib, 10 μ M) inhibit the activity of Lyn kinase, and quercetin can reduce the activation of Lyn kinase by Lyn agonist (Tolimidone, 10 μ M). We thus reveal the potential of quercetin to reduce allergic conjunctivitis through the inhibition of Lyn activity.

2. Material and methods

2.1. Drugs and reagents

Quercetin was purchased from Baojichenguang (Baoji, China) and purified to $\geq 98\%$ (The 2% impurities have not been characterized). Olopatadine eye drops was purchased from Baojichenguang (Baoji, China). Dimethyl sulfoxide (DMSO), anti-dinitrophenol (DNP)-specific IgE, DNP-HSA, albumin from chicken egg white (OVA), and Aluminum hydroxide were procured from Sigma Aldrich Co., LLC. (St. Louis, MO, USA). Saline was purchased from Shandong Qidu Pharmaceutical Co., Ltd. (Shandong, China). P-nitrophenyl N-acetyl- β -D-glucosamide and Triton X-100 were purchased from Sigma Aldrich Co., LLC. (Shanghai, China). HA was purchased from sigma, and HA-2HCl (A, A, B, B-D $_4$, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA). LCMS 8040 mass spectrometer was purchased from Shimadzu Corporation, Kyoto, Japan. HILIC column (Venusil HILIC, 2.1 mm $\times$ 150 mm, 3 μ m) was obtained from Agela Technologies, Tianjin, China. HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, USA). Mass spectrometry grade formic acid was purchased from Sigma. Human MCP-1 (EH019-96), Human TNF- α (EH009-96), Human IL-8 (EH005-96), Human IL-13 (EH024-96) ELISA Kits were purchased from EXCELLBIO CO., LTD. (Shanghai, China). Mouse IgE, substance P, TNF- α (EM008-96) and IL-4 ELISA Kits were purchased from EXCELLBIO CO., LTD. (Shanghai, China). Fluo-3, AM ester and Pluronic F-127 were purchased from Biotium. Substance P was purchased from Santa Cruz Biotechnology; Bovine serum albumin were purchased from GenDepot, Katy, TX, USA. Normal serum were purchased from Jackson Immuno Research; NiCl $_2$ $\cdot$ 6H $_2$ O were purchased from Sigma. Bafetinib and Tolimidone were purchased from MCE (MedChemExpress).

TM buffer (6.954 g/l NaCl, 0.353 g/l KCl, 0.282 g/l CaCl $_2$, 0.143 g/l MgSO $_4$, 0.162 g/l KH $_2$ PO $_4$, 2.383 g/l HEPES, 0.991 g/l glucose and 1 g/l BSA, pH = 7) was prepared on the day of experiments. CIB (NaCl

Table 1
Modified post-signal scoring criteria.

Score Symptoms	0	1	2	3
Conjunctival hyperemia	nothing	Blood vessel bright red	Blood vessel dark red	Diffuse purple
Conjunctival edema	nothing	slight	deep	epicontinental
Eyelid edema	nothing	Minor/eyelid valgus	Semi-closed	More than half closed
Secretion	nothing	little	Eyelids hair damp	wetness throughout the week

125 mM, KCl 3 mM, CaCl₂ 2.5 mM, MgCl₂ 0.6 mM, HEPES 10 mM, glucose 20 mM, NaHCO₃ 1.2 mM, sucrose 20 mM, brought to pH 7.4 with NaOH) was prepared on the day of experiments. Stop buffer consisted of 0.1 M sodium carbonate and sodium bicarbonate (pH 11). All aqueous solutions were prepared in ultrapure water (MK-459 Millipore Milli-Q Plus ultra-pure water system).

2.2. Cell lines and C57BL/6 mice

The Laboratory of Allergic Disease 2 (LAD2) human MCs were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). Cells were maintained in StemPro-34 medium supplemented with 10 ml/l StemPro nutrient supplement, 1:100 penicillin-streptomycin, 2 mmol/l L-glutamine and 100 ng/ml human stem cell factor. Cells were maintained in 5% CO₂ at 37 °C. Culture medium was replaced every week and cells were maintained at a density of 2×10^6 cells/ml. C57BL/6 mice were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Adult male mice weighing 25–30 g were included in the study.

2.3. Animal model preparation

OVA (100 µg) was administered every day through intraperitoneal injection in an adjuvant Al (OH)₃ mixture (5 mg, 200 µl) for 3 weeks. Then, the right eyes were washed in 100 µl of normal saline and OVA (400 µg) was instilled into the eye every day for 7 d. After 72 h, 100 µl of OVA (400 µg, pH 7.2) was instilled into the eye to induce allergen challenge and AC symptoms. Once the model was established, low, medium and high doses of quercetin (1, 2, 4 mg/kg) (0.025–0.03 µg, 0.05–0.06 µg and 0.10–0.12 µg for each mice) and olopatadine (10 µl) were administered by gavage according to body weight continuously for 7 days. Control groups were administered equivalent amounts of saline by gavage. The volume of the gavage was 10 ml/kg. Animals were not anesthetized during dosing.

At the end of the treatment period, OVA was instilled into the right eye. The AC hypersensitivity reaction was recorded 20 min post-challenge. After 24 h, the left eyeball method was used to collect blood, and animals were then sacrificed. Serum were removed for detecting the expression of IgE, HA, IL-4, TNF-α and substance-P. The right eye conjunctiva were used for pathological observations such as eosinophil numbers, MC degranulation, vascular permeability and substance P concentrations. The right eye conjunctiva were also used for RT-PCR to assessment the mRNA expression of IL-4 and TNF-α.

2.4. Test group

Model mice were randomly divided into model, quercetin treatment (including high, medium and low doses), comparator group (olopatadine eye drops, a comparator to quercetin for the treatment of allergic conjunctivitis), saline control, and normal control groups. Each group consisted of 12 mice (n = 84). The model group consisted of AC model mice with normal feeding; the quercetin low, medium and high dose groups consisted of AC model mice administered 1 mg/kg, 2 mg/kg, and 4 mg/kg quercetin respectively, by stomach every day; the olopatadine group consisted of AC model mice administered 5 µl 0.1% HCl olopatadine eye drops (Bid) into the eye every day; the saline control

group consisted of AC model mice administered an equal volume of saline into the stomach every day; the normal control group given no treatment.

2.5. Signature criteria

The ocular signs of allergic conjunctivitis were observed under a slit lamp microscope 20 min after challenge with OVA. Blind methods were used to score different degrees of conjunctival edema, conjunctival hyperemia, eyelid edema, and secretions by clinical ophthalmologists (0–3 points). Finally, the scores of the four indicators were added to obtain the total score, with a minimum of 0 points and a maximum of 12 points. The scoring standard refers to the Magone (Teresa Magone Chi-Chao et al, 1998) standard. The improved eye scoring standards are shown in Table 1 (Li Gao et al, 2012).

2.6. Serum preparation

Blood was collected from the left eyeball into centrifuge tubes and stored at low temperatures. Blood were centrifuged at 3000 g/min for 15 min at 4 °C, plasma was pipetted into small centrifuge tubes and immediately stored at −20 °C. Plasma were slowly thawed in ice-water prior to assessments.

2.7. Eye specimen preparation

Mice were sacrificed by exsanguination after 24 h of stimulation. The fur was removed from the gingival margin, and the contents of the eyelids and eyeball were collected, leaving only the eyelids, conjunctiva and eyeballs. The samples was fixed in 10% neutral formaldehyde solution and dehydrated in an ethanol gradient after 24 h. The samples was immersed in wax, embedded into paraffin blocks, and serially sectioned at 5 µm. Hematoxylin-eosin staining (HE), toluidine blue staining; Substance P immunohistochemistry were performed.

2.8. Hematoxylin-eosin staining (HE)

The tissue was embedded in paraffin and cut into 5 µm. The paraffin section was dewaxed by treatment with xylene I for 20 min, xylene II for 20 min, anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min and 75% alcohol for 5 min, and then washed with tap water. The sections were stained with hematoxylin staining solution for 3–5 min, then washed with tap water, separated for liquid differentiation, washed with tap water, then returned to blue with blue back liquid, and rinsed with tap water. The sections were sequentially dehydrated with 85% and 95% gradient alcohol for 5 min and stained with eosin dye solution for 5 min. After staining, the sections were made transparent with xylene and then sealed with a neutral gel. Microscopic examination, image acquisition and analysis showed that the nucleus was blue and the cytoplasm was red.

2.9. Toluidine blue staining

The tissue was embedded in paraffin and sectioned to 5 µm. Paraffin sections were routinely dewaxed to water, and then stained with 1% toluidine blue solution for 5 min–8 min (stain solution preparation: 1 g

of toluidine blue dissolved in 100 ml of distilled water, 2 ml of mordant added dropwise, placed in a 60 °C incubator for 2 h, then moved to room temperature for use) and then washed by distilled water, 95% ethanol rapidly differentiated; anhydrous ethanol dehydrated, xylene transparent neutral gum seal. Microscopic examination, image acquisition.

2.10. Immunohistochemical staining

AC tissue was tested by immunohistochemical staining. AC tissue sections were washed three times with PBS for 5 min, then reacted with 0.3% H₂O₂ for 10 min. Tissue was reacted for 1 h in blocking solution, which was a mixture of PBS with 10% normal serum. The tissue was then washed three times, and treated with primary antibody overnight at 4 °C. Substance P were used as the primary antibodies in a solution with a mixture of bovine serum albumin and Triton X-100 with PBS. After reaction, the tissue was incubated with peroxidase IgG for 1 h. It was then reacted with diaminobenzidine tetrachloride mixed with NiCl₂ · 6 H₂O. Finally, the tissue was stained with Methyl Green as a counter stain.

2.11. Chemokine release assays

AC mice serum were collected, the assays of mouse IgE, IL-4, TNF-α and substance P were performed according to the manufacturer's instructions.

2.12. Cytotoxicity assays

LAD2 cells were incubated with IgE (1 µg/ml) overnight and cell viability was determined using the 7 Sea-Cell Counting Kit according to the manufacturer's recommendations (7 Sea Pharmatech Co., Ltd). Briefly, LAD2 cells were seeded into 96-well plates (5 × 10³ cells/well) and treated with 0, 50, 100, 200, and 400 µM of quercetin for 24 h. Cells were then treated with 7 Sea-Cell Counting Kit solution (10 µl) for 2.5 h and relative cell viability was assessed on a microplate reader (Bio-Rad, Carlsbad, CA, USA) at 450 nm. Inhibition rates were calculated in Graphpad Prism 7 software.

2.13. EC₅₀ assays

LAD2 cells were incubated in IgE (1 µg/ml) overnight and plated into 96-well plates at a density of 2 × 10⁴ cells per well. The culture medium was removed and quercetin (0, 12.5, 25, 50, 100, 200, 400, 800 µM) in combination with DNP-HSA (1 µg/ml) were added to cells for 30 min at 37 °C. The release rates of hexosaminidase were then calculated (see the methods of *β*-hexosaminidase assays). EC₅₀ values were assessed using Prism 7 software.

2.14. Intracellular Ca²⁺ mobilization assay

LAD2 cells were incubated with IgE (1 µg/ml) overnight and drugs were diluted to the required concentrations in Ca²⁺ imaging buffer (CIB). The incubation buffer consisted of 0.5 µl Fluo-3, 2 µl Pluronic F-127 and 997.5 µl CIB. LAD2 cells were washed twice in CIB and plated at a density of 1 × 10⁴ cells per well in 96-well plates. For Ca²⁺ imaging, cells were magnified 200 × and one photo per second was taken under blue light. Cells were identified as responsive if the [Ca²⁺] increased by at least 50% post-injection. DNP-HSA (1 µg/ml) was used as a positive control.

2.15. β-hexosaminidase assays

LAD2 cells were treated with IgE (1 µg/ml) overnight in 96-well plates. Culture medium was then removed and quercetin was added at the indicated concentrations in TM buffer for 30 min at 37 °C. DNP-HSA

(1 µg/ml) was added to cells in TM buffer for 30 min and the plates centrifuged at 2000 g for 5 min at 25 °C. For the analysis of total β-hexosaminidase content, cells were lysed in 0.1% Triton X-100 in TM buffer. B-hexosaminidase levels were quantified through the hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosamide in 0.1 M citric acid/sodium citrate buffer (pH = 4.5) for 90 min at 37 °C. The reaction was stopped through the addition of stop buffer (0.1 M sodium carbonate/sodium bicarbonate pH = 11). The percentage of β-hexosaminidase release was assessed at an absorbance of 405 nm using a spectrophotometer as follows: absorbance of culture supernatants at 405 nm × 100/absorbance of total cell lysate supernatants at 405 nm. DNP-HSA (1 µg/ml) was used as a positive control.

2.16. Histamine release assays

LAD2 cells were treated with IgE (1 µg/ml) overnight. In an LCMS 8040 mass spectrometer, HA was evaluated on the system using a HILIC column and isocratic elution was performed using acetonitrile-water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 ml/min. DNP-HSA (1 µg/ml) was used as a positive control.

2.17. Chemokine release assays

LAD2 cells were treated with IgE (1 µg/ml) overnight and plated into 96-well plates at a density of 1 × 10⁶ cells per well. The culture medium was removed and quercetin in combination with DNP-HSA (1 µg/ml) was added at the indicated concentrations for 6 h. The assays of human IL-13, human MCP-1, human IL-8, human TNF-α were performed according to the manufacturer's instructions. DNP-HSA (1 µg/ml) was included as a positive control.

2.18. Western blot analysis

LAD2 cells were treated with IgE (1 µg/ml) overnight and incubated with medium (control) or quercetin (0, 25, 50 and 100 µM) alone or in combination with DNP-HSA (1 µg/ml) for 24 h. Following incubation, cells were washed twice in cold PBS and lysed. Proteins were extracted using a Total Protein Extraction Kit. Lysates were centrifuged at 12,000 g at 4 °C for 20 min. BCA Protein Assays (Thermo Fisher Scientific, Rockford, USA) were used to quantify protein concentrations. Lysates were immunoblotted with rabbit anti-human antibodies including: anti-Lyn (1:1000, #2796, Cell Signaling Technology [CST]), anti-phospho-Lyn (1:1000, #2731, CST); anti-PLCγ1 (1:1000, #5690, CST), anti-phospho-PLCγ1 (p-PLCγ1, Ser1248, 1:1000, #8713, CST), anti-IP3R (1:1000, #3763, CST), anti-phospho-IP3R (p-IP3R, Ser1756, 1:1000, #8548, CST), anti-ERK1/2 (1:1000, p44/42 MAPK (Erk1/2), #9102, CST), anti-phospho-ERK1/2 (p-Erk1/2, p-p44/42 MAPK (Erk1/2) [Thr202/Tyr204], 1:1000, #9101, CST), anti-NF-κB (p65, 1:1000, ab7970, Abcam), anti-phosphorylated-IKK-α/β (1:1000, #2697L, CST) or anti-GAPDH (1:2000, #2118, CST). Enhanced chemiluminescence was used to visualize proteins. GAPDH was used as loading control. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the protein levels.

2.19. Statistical analysis

Group data are expressed as the means ± standard deviation (SD) from 5 or more independent experiments. Independent sample variance analysis was used to determine significance in statistical comparisons using SPSS. Differences were considered significant at *p* < .05 (**p* < .05, ***p* < .01, ****p* < .001 and *****p* < .0001).

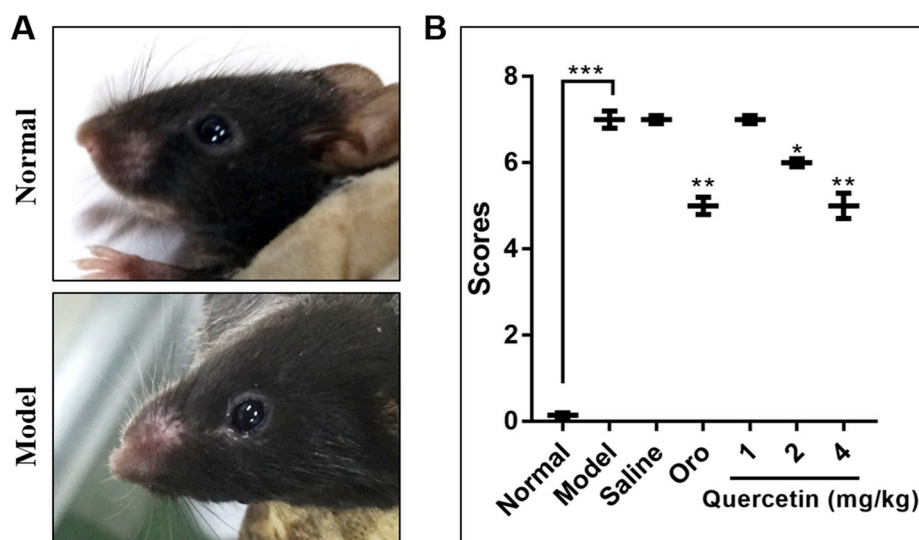


Fig. 1. Clinical and histopathological scores.

(A) Eye features of normal mice and model mice. (B) Eye signs score of mice. The data are presented as mean ± S.D. (n = 12). One-way ANOVA (Bonferroni's multiple comparisons test) was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

3. Results

3.1. Clinical and histopathological scores

We successfully induced allergic conjunctivitis using OVA in C57BL/6 mice. Clinical and histopathological scores were consistent with the successful establishment of the model. Clinical and histopathological scores were: 0 (control), 7 (model group), 5 (Oro), 7 (quercetin, 1 mg/kg), 6 (quercetin, 2 mg/kg), and 5 (quercetin, 4 mg/kg) in C57BL/6 mice (Fig. 1A and B). The release of IgE (Fig. 2A), HA (Fig. 2B), IL-4 (Fig. 2C), and TNF- α (Fig. 2D) in model mice increased compared to normal controls. The mRNA expression of IL-4 (Fig. 2E) and TNF- α (Fig. 2F) significantly increased in model mice compared to normal controls. Serum analysis revealed increased concentrations of substance P (Fig. 2G) in the model mice compared to normal controls. Histological analysis revealed inflammatory processes in the conjunctiva and cornea, epithelial lesions, edema, infiltration, and a

predominance of eosinophils (Fig. 3A and B), increased MC dispersion (Fig. 3C and D) and vascular permeability (Fig. 3E and F) in the conjunctival tissue. Substance P expression was rarely detected in normal mice. Compared to the control group, substance P levels were significantly higher in the model group, evidenced by the increased levels of staining and expanded stained areas (Fig. 3G and H). The mouse conjunctivitis model was thus successfully established based on these multiple lines of verification.

3.2. Effects of quercetin on inflammatory factors in allergic conjunctivitis C57BL/6 mice

Through studying the effects of quercetin on blood inflammatory factors including IgE, HIS, IL-4 and TNF- α in mouse models, inflammatory factor expression decreased after intervention with quercetin and olopatadine eye drops. Compared to AC mouse model (saline group), quercetin inhibited the secretion of IgE (Fig. 4A), HA (Fig. 4B),

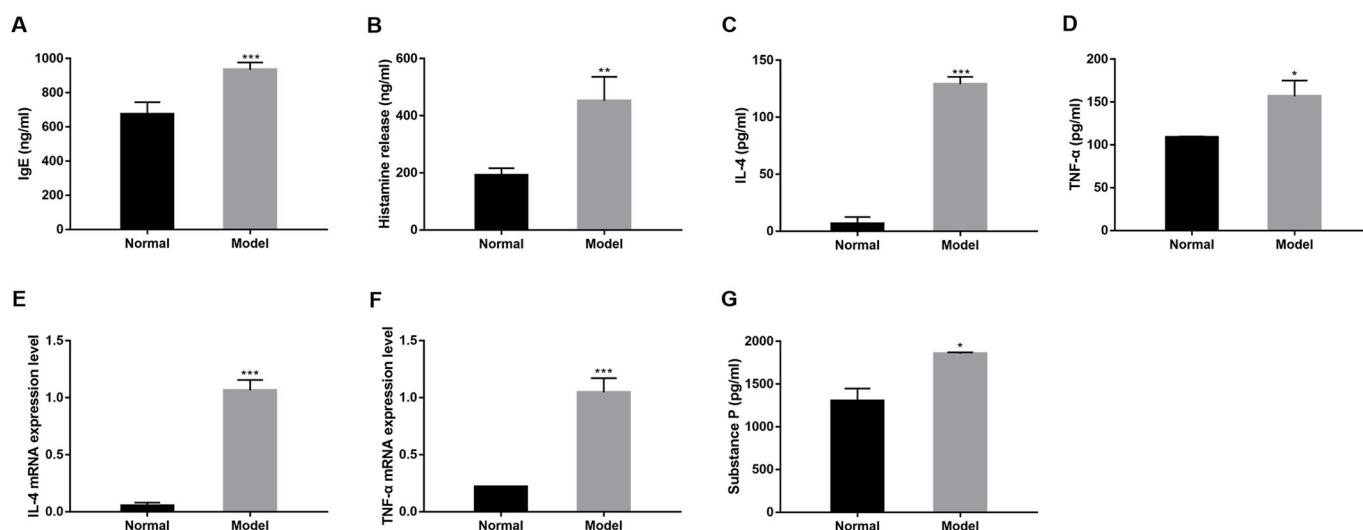


Fig. 2. Measurement of cytokine levels in the supernatant from normal and model mice.

(A) The IgE release of normal and AC model mice. (B) The histamine release of normal and AC model mice. (C) The IL-4 release of normal and AC model mice. (D) The TNF- α release of normal and AC model mice. (E) The level of IL-4 mRNA in normal and AC model mice. (F) The level of TNF- α mRNA in normal and AC model mice. (G) The Substance P release of normal and AC model mice. Compared with the normal mice, chemokine secretion increased in AC model mice were detected with an ELISA array. The data are presented as mean ± S.D. (n = 5). Two-tailed unpaired Student's t-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

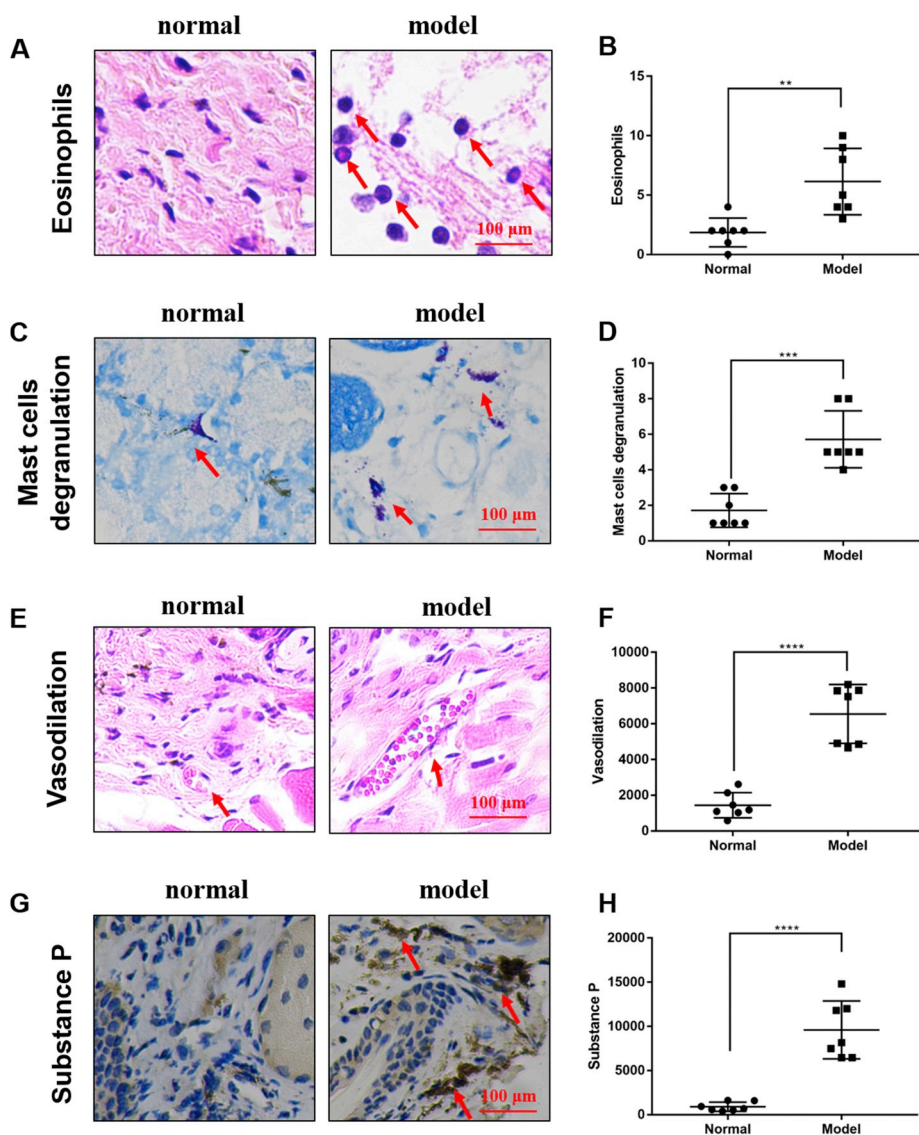


Fig. 3. Histopathologic analysis of normal and model mice.

(A) Compared with the normal mice, eosinophil number increased in AC model mice, demonstrated by H&E staining of skin tissue sections. (B) Quantification of eosinophil expression in (A) by densitometric analysis ($n = 7$). (C) Compared with the normal mice, mast cells degranulation increased in AC model mice, demonstrated by toluidine blue staining of skin tissue sections. (D) Quantification of mast cells expression in (C) by densitometric analysis ($n = 7$). (E) Compared with the normal mice, hemangiectasis increased in AC model mice, demonstrated by H&E staining of skin tissue sections. (F) Quantification of hemangiectasis in (E) by densitometric analysis ($n = 7$). (G) Compared with the normal mice, Substance P concentrations increased in AC model mice, demonstrated by immunohistochemistry of skin tissue sections. (H) Quantification of Substance P expression in (G) by densitometric analysis ($n = 7$). All experiments were repeated at least three times ($n = 7$). Data are expressed as mean $\pm$ SD, and analyzed using two-tailed unpaired student's t-test. Statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

IL-4 (Fig. 4C) and TNF- α (Fig. 4D) in a concentration-dependent manner in AC mouse model and the effect is similar to olopatadine (positive drug group). Collectively, these data demonstrate that quercetin inhibits the release of inflammatory cytokines in allergic conjunctivitis mouse models.

Compared to AC mouse model (saline group), the mRNA expression of IL-4 (Fig. 4E) and TNF- α (Fig. 4F) in the conjunctival tissue were significantly lower after intervention with low, medium and high doses of quercetin and the effect is similar to olopatadine (comparator drug group). This suggested that quercetin can inhibit the release of inflammatory cytokines in allergic conjunctivitis mice.

3.3. Histopathological analysis

The levels of eosinophils were determined by HE staining. In the model group, the eosinophils levels significantly increased. Following quercetin or olopatadine treatment, the levels of eosinophils decreased and the effect is similar to olopatadine (comparator drug group) (Fig. 5A and B). The degranulation of MCs was determined by toluidine blue staining in mice conjunctiva. In the model group, conjunctival MCs

were aggregated and degranulated. Following quercetin or olopatadine intervention, both MC number and the rate of degranulation decreased and the effect is similar to olopatadine (comparator drug group) (Fig. 5A, C). Vascular permeability (VP) was determined by HE staining. Compared to AC mouse model (saline group), VP significantly decreased following quercetin or olopatadine treatment (Fig. 5A, D) and the effect is similar to olopatadine (comparator drug group). This confirmed that quercetin could inhibit inflammatory processes in AC mouse mice.

3.4. Effects of quercetin on substance P in allergic conjunctivitis C57BL/6 mice

The substance P concentrations were determined by substance P ELISA kit. Compared to AC mouse model (saline group), the substance P concentration significantly decreased following quercetin and olopatadine treatment (Fig. 4G) and the effect is similar to olopatadine (comparator drug group).

The substance P concentrations were determined by immunohistochemistry in which conjunctival expression was yellow/tan

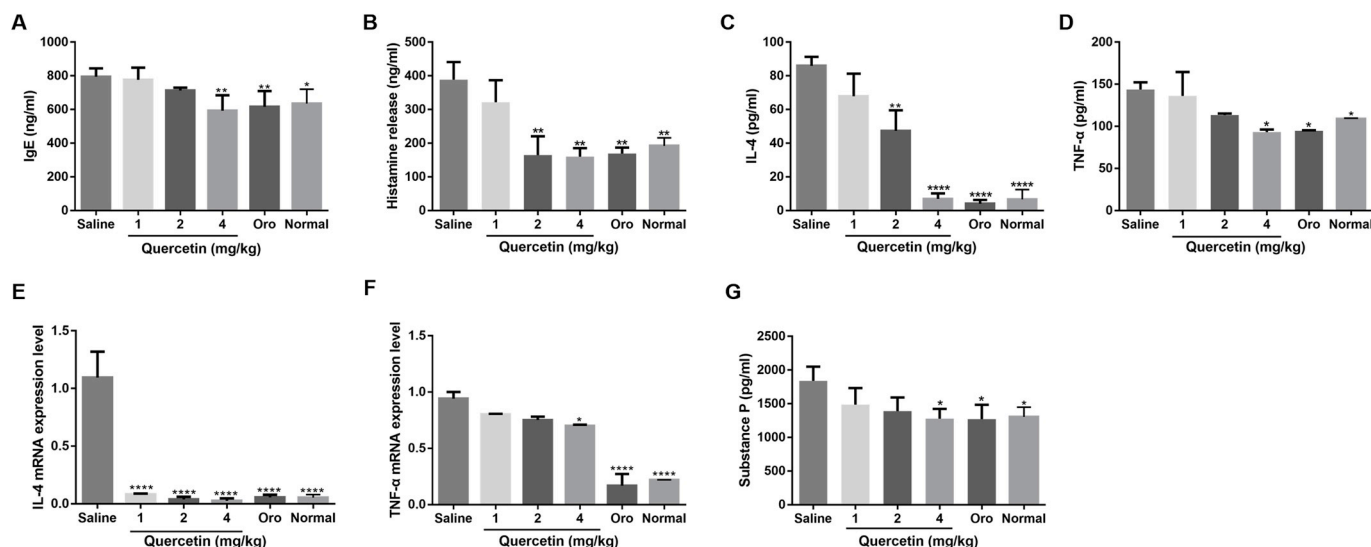


Fig. 4. Measurement of cytokine levels in the supernatant from model mice.

(A) The IgE release of saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice. (B) The histamine release of saline group, quercetin (1, 2, 4 mg/kg) group and Oro group in AC model mice. (C) The IL-4 release of saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice. (D) The TNF-α release of saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice. (E) The level of IL-4 mRNA in saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice. (F) The level of TNF-α mRNA in saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice. (G) The Substance P release of saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice. Compared with the saline group, chemokine secretion decreased in quercetin (1, 2, 4 mg/kg) group and Oro group were detected with an ELISA array. The data are presented as mean ± S.D. (n = 5). One-way ANOVA (Bonferroni's multiple comparisons test) was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$).

coloured when imaged using a 200 x optical microscope. Compared to AC mouse model (saline group), the substance P concentration decreased in each treatment group (quercetin and olopatadine) in a dose-dependent manner and the effect is similar to olopatadine (comparator drug group) (Fig. 5A, E). This suggested that quercetin inhibits inflammatory processes through regulating the expression of substance P in AC mouse mice.

3.5. Effects of quercetin on LAD2 cells

The effects of quercetin on the viability of LAD2 cells was assessed using 7 Sea-Cell Counting Kits. No effects on cell viability were observed when cells were treated with 200 μM quercetin (Fig. 6A). Apoptosis assays revealed that 24 h treatment with 25, 50, 100 and 200 μM quercetin produced no cytotoxic effects, whilst 400 μM quercetin had modest effects on LAD2 cell health (Fig. 6D). Quercetin had no effects on Ca^{2+} influx (Fig. 6C). It was thus concluded that quercetin does not influence LAD2 viability or metabolic processes.

3.6. Quercetin attenuates DNP-HSA/IgE-triggered Ca^{2+} influx and chemokine release in LAD2 cells

Ca^{2+} influx mediates MC degranulation and HA release. Quercetin inhibited DNP-HSA/IgE triggered Ca^{2+} influx in LAD2 MCs in a concentration-dependent manner (0, 25, 50, 100 μM) (Fig. 7A and B) with a half-maximal effective concentration (EC_{50}) of 79.4 ± 26.9 ng/ml (Fig. 6B). To verify if quercetin can suppress DNP-HSA/IgE induced MC activation through its ability to prevent degranulation and chemokine release, β-hexosaminidase secretion and HA, IL-13, MCP-1, IL-8 and TNF-α levels were assessed. Quercetin inhibited the secretion of both β-hexosaminidase (Fig. 7C) and HA (Fig. 7D) following DNP-HSA treatment. Compared to DNP-HSA alone, quercetin inhibited the secretion of IL-13 (Fig. 7E), MCP-1 (Fig. 7F), IL-8 (Fig. 7G) and TNF-α (Fig. 7H) in a

concentration-dependent manner. Taken together, these data highlight the ability of quercetin to inhibit DNP-HSA/IgE induced Ca^{2+} influx, MC degranulation, and chemokine release.

3.7. Quercetin inhibits Lyn-PLCγ signaling and regulates Ca^{2+} levels in mast cells

In the experiments that followed, LAD2 cells were incubated with various concentrations of quercetin (0, 25, 50, 100 μM) alone or in combination with DNP-HSA (1 μg/ml) for 24 h, and its effects on inflammatory signaling assessed. Quercetin inhibited DNP-HSA/IgE induced Lyn phosphorylation (Fig. 8A and B), PLCγ-IP3R- Ca^{2+} signaling (Fig. 8C and D), ERK1/2 phosphorylation (Fig. 8E and F), IKK phosphorylation and NF-κB expression (Fig. 8G and H) in a concentration dependent manner. Quercetin treatment (0, 25, 50, 100 μM) alone had no effects on PLCγ1 expression (Fig. 8K and L), but reduced the expression of total and phosphorylated Lyn (Fig. 8I and J). Quercetin (200 μM) and Lyn inhibitors (Bafetinib, 10 μM) inhibit the activity of Lyn kinase, and quercetin can reduce the activation of Lyn kinase by Lyn agonist (Tolimidone, 10 μM) (Fig. 8M and N). It is therefore likely that quercetin is a potent Lyn kinase inhibitor.

4. Discussion

The animal model of AC is divided into active and passive immunization. The former uses an allergen to directly induce an allergic reaction with an agonist, and the latter directly stimulates the reaction with an immune serum. Active immunization is widely used because it is closer to the natural pathogenesis. Animals most frequently used in these studies are mice, guinea pigs or rats. In this experiment, egg albumin (active immunization mice) was used to establish an animal model. Previous experiments (Li Gaoet al, 2012) have confirmed that the model can be highly simulated and stable and reliable. Therefore,

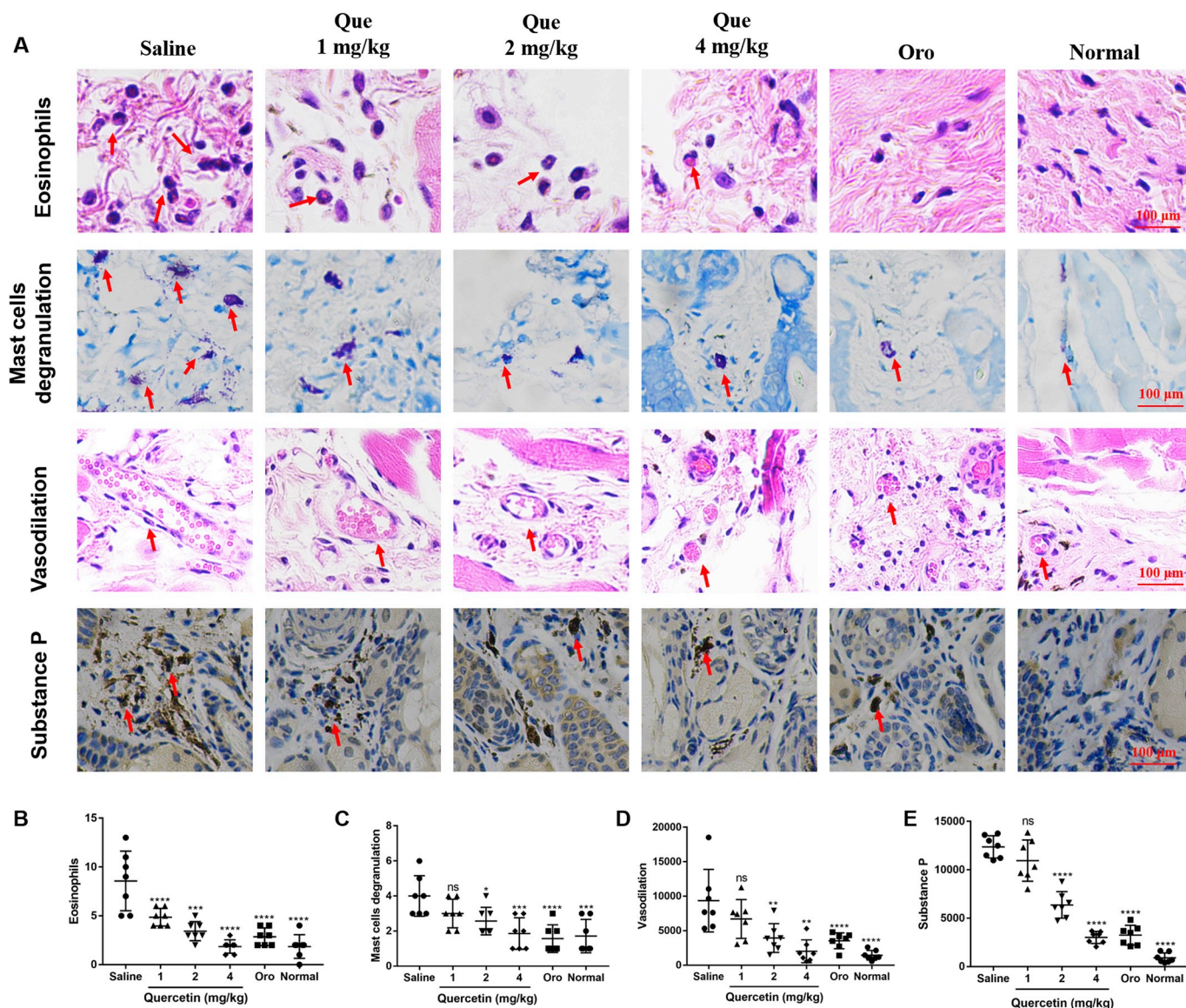


Fig. 5. Histopathologic analysis of eosinophil, mast cells degranulation, hemangiectasis and substance P.

(A) The eosinophil number of saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice by H&E staining of skin tissue sections; The mast cells degranulation of saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice by toluidine blue staining of skin tissue sections; The hemangiectasis of saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice by H&E staining of skin tissue sections; The Substance P concentrations of saline group, quercetin (1 mg/kg, 2 mg/kg, 4 mg/kg) group and Oro group in AC model mice by immunohistochemistry of skin tissue sections. (B) Quantification of eosinophil expression in (A) by densitometric analysis ($n = 7$). All experiments were repeated at least three times ($n = 7$). (C) Quantification of mast cells expression in (A) by densitometric analysis ($n = 7$). (D) Quantification of hemangiectasis in (A) by densitometric analysis ($n = 7$). All experiments were repeated at least three times ($n = 7$). (E) Quantification of Substance P expression in (A) by densitometric analysis ($n = 7$). Data are expressed as mean $\pm$ SD. and analyzed using One-way ANOVA (Bonferroni's multiple comparisons test). Statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

we used this AC model to investigate the effect of quercetin on conjunctivitis.

In this study, we successfully established an allergic conjunctivitis C57BL/6 mouse model using OVA. The model mirrored the symptoms of humans both clinically and histopathologically. The mice rubbed their eyes, showed eyelid and conjunctival edema, eye secretions, and conjunctival congestion, similar to human allergic reactions (Friedlaender, 1993). Histopathological analysis revealed neutrophil and eosinophilic infiltration and the degranulation of MCs in the

conjunctiva (Bonini et al., 1988). In addition to eye disease, systemic immune responses are induced by OVA. Under our laboratory conditions, the production of IgE, HA, IL-4, TNF- α , and substance P increased in the model groups. Having established the model, we assessed the effects of quercetin on allergic conjunctivitis, including its ability to inhibit the release of MC factors. We further assessed the effects of quercetin on known inflammatory signaling pathways in primary human MCs to reveal the molecular mechanisms responsible for its effects.

The proinflammatory mediators IL-4 and TNF- α are produced by the

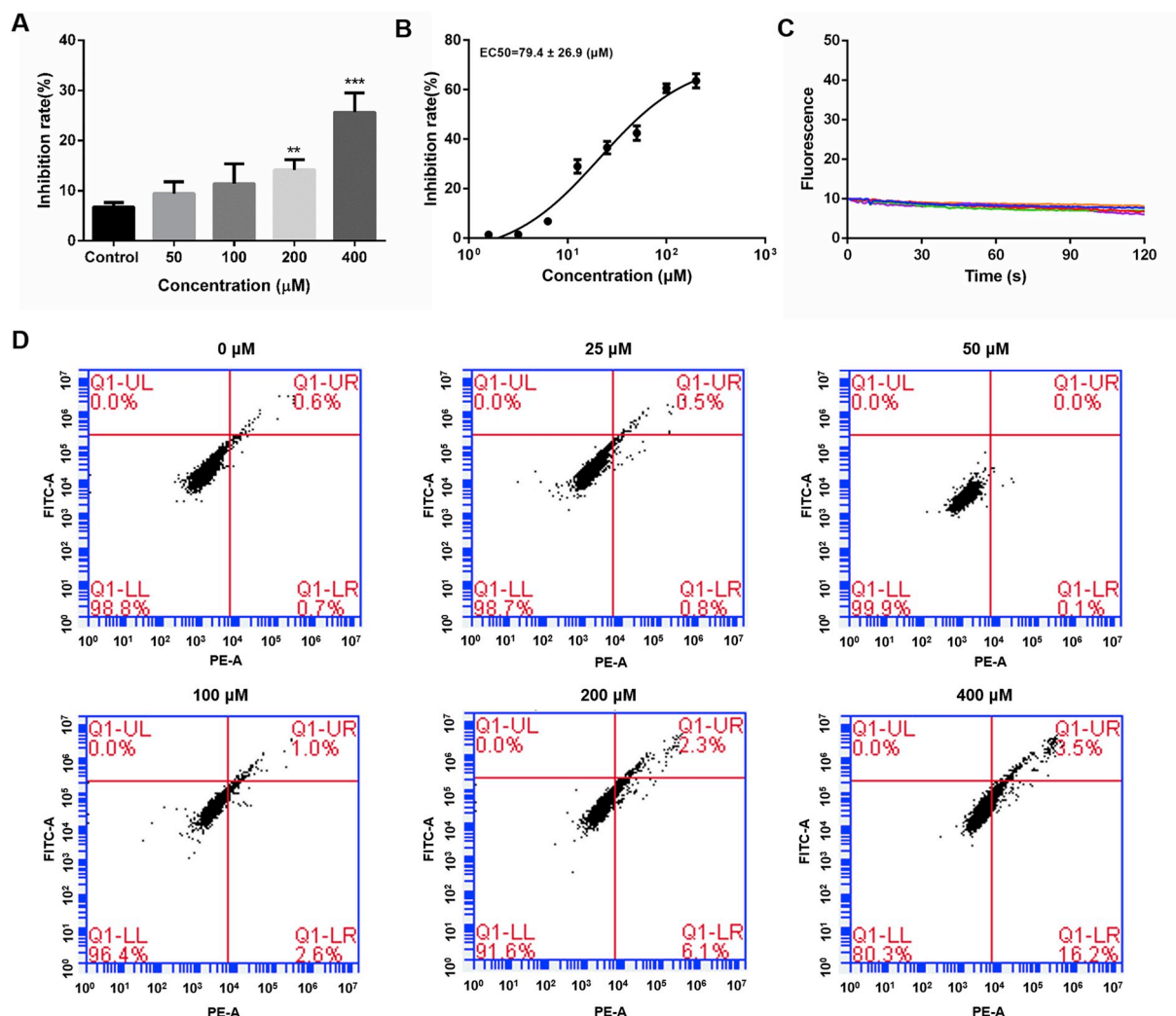


Fig. 6. Effect of quercetin on human mast cells.

(A) The cytotoxicity in LAD2 cells treated with 0, 50 μM, 100 μM, 200 μM, 400 μM quercetin. (B) The EC₅₀ in LAD2 cells treated with 0, 25 μM, 50 μM, 100 μM quercetin or 1 μg/ml DNP-HSA for 45 min. (C) Representative imaging traces Ca²⁺ concentrations treated by 100 μM quercetin in LAD2 cells. (D) The cell apoptosis treatment with different concentrations of quercetin (0, 25, 50, 100, 200 and 400 μM). The data are presented as mean ± S.D. (n = 5). One-way ANOVA (Bonferroni's multiple comparisons test) was used to determine significance in statistical comparisons, and statistical significance was accepted at p < .05 (**p < .01, ***p < .001).

degranulation of MCs and have important biological roles in AC (Leonardi, 2002; Theoharides and Kalogeromitros, 2006). Studies have shown that IL-4 and TNF-α promote and maintain the allergic response. IL-4 increased the production of IgE in B-cells (Kuhn et al., 1991) and promoted T-cell differentiation into Th2 cells (Hines, 2002). TNF-α promotes the chemotaxis of eosinophils, neutrophils, and giant sputum cells, increasing cytotoxicity, and promoting the release of MC mediators (Hines, 2002). In the allergic conjunctivitis C57BL/6 model, we focused on the degranulation of MCs during disease pathogenesis and explored the beneficial effects of quercetin. We found that quercetin reduces the MC number in conjunctival tissue, inhibits the release of IgE, HA, IL-4 and TNF-α into peripheral blood, and reduces the mRNA expression of IL-4 and TNF-α in conjunctival tissue. Quercetin could also inhibit the aggregation and degranulation of mice MCs and effectively inhibit the pre-synthesis of HA in MCs. Quercetin could therefore stabilize MCs, reduce their degranulation, and prevent the release of inflammatory mediators.

Substance P is a neuropeptide enriched in conjunctiva immunoreactive nerve fibers (Selbach et al., 2005). In our AC model, the mouse conjunctival tissue had significantly higher levels of substance P, manifested by more intense staining and area enlargement. Quercetin inhibited the increase of substance P concentrations in the conjunctival tissue in a dose-dependent manner. Previous studies reported that the anti-IgE activation of hCBMCs increases intracellular Ca²⁺ levels which is a necessary step for the release of inflammatory mediators (Kempuraj et al., 2003; Saito et al., 1996). Fewtrell & Gomperts showed for the first time that quercetin inhibits Ca²⁺ influx in rat peritoneal MCs (Fewtrell and Gomperts, 1977). The IgE-mediated release of HA and leukotrienes is eliminated following Ca²⁺ depletion (Kimata et al., 1999). Quercetin also inhibits ionophore-induced HA release from rat peritoneal MCs, suggesting that it has receptor-mediated effects other than Ca²⁺ influx (Ennis et al., 1981). Quercetin inhibits the production of HA, IL-6, and TNFα in cultured murine MCs from bone marrow-derived cells (Kimata et al., 2000). Quercetin also inhibits connective tissue and mucosal MCs

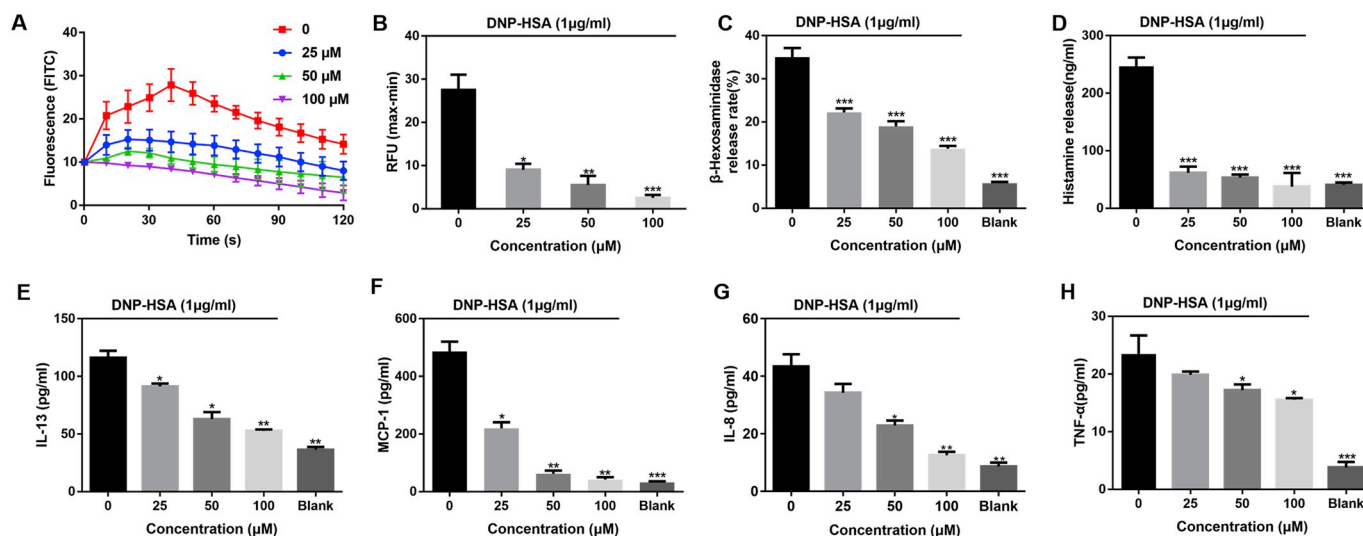


Fig. 7. Quercetin attenuated calcium flux and chemokines release in LAD2 cells.

(A) Representative imaging traces Ca^{2+} concentrations treated by 1 $\mu\text{g/ml}$ DNP-HSA and 0, 25, 50, 100 μM quercetin in LAD2 cells. (B) Quantification of responding cells of DNP-HSA. Scale bar, 10 mm. (C) The β -hexosaminidase release in LAD2 cells treated with 0, 25, 50, 100 μM Quercetin combined with 1 $\mu\text{g/ml}$ DNP-HSA for 45 min. (D) The histamine release in LAD2 cells treated with 0, 25, 50, 100 μM quercetin combined with 1 $\mu\text{g/ml}$ DNP-HSA for 45 min. (E) The IL-13 release in LAD2 cells treated with 0, 25, 50, 100 μM quercetin combined with 1 $\mu\text{g/ml}$ DNP-HSA for 45 min. (F) The MCP-1 release in LAD2 cells treated with 0, 25, 50, 100 μM quercetin combined with 1 $\mu\text{g/ml}$ DNP-HSA for 45 min. (G) The IL-8 release in LAD2 cells treated with 0, 25, 50, 100 μM quercetin combined with 1 $\mu\text{g/ml}$ DNP-HSA for 45 min. (H) The TNF- α release in LAD2 cells treated with 0, 25, 50, 100 μM quercetin combined with 1 $\mu\text{g/ml}$ DNP-HSA for 45 min. The data are presented as mean $\pm$ S.D. ($n = 5$). One-way ANOVA (Bonferroni's multiple comparisons test) was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

in rats (Pearce et al., 1984), and HA secretion in human lung and intestinal MCs (Fox et al., 1988). In this study, quercetin effectively prevented the increase in intracellular Ca^{2+} in stimulated human MCs. Quercetin attenuated the release of β -hexosaminidase, HA, MCP-1, TNF- α , IL-13 and IL-8 in LAD2 cells treated with DNP-HSA, confirming its ability to inhibit DNP-HSA/IgE induced Ca^{2+} influx, MC degranulation, and chemokine release.

Fc ϵ RI signaling is triggered by the IgE mediated cross-linking of the tetrameric Fc ϵ RI complex, which includes Lyn, Syk, PLC γ and PKC, leading to an allergic reaction (Yamamoto et al., 2003). Fc ϵ RI signaling is triggered by Syk and Lyn phosphorylation which act as docking sites for aptamer molecules including Gads, Grb2, SLP76, PLC γ and PKC, which in turn activate downstream signaling cascades (Wong et al., 2004). It has been reported that the regulation of Syk is a primary target for the treatment of allergic disease. For example, nebulized Syk antisense oligonucleotides are used as therapeutic agents for allergic rhinitis and asthma (Stenton et al., 2002). To determine the mechanism of quercetin activity, we examined its effects on inflammatory signaling. Quercetin inhibited the phosphorylation of a variety of signaling proteins including Lyn, PLC γ , and IP3R. These results indicated that quercetin has anti-allergic effects in antigen-stimulated MCs through the inactivation of Fc ϵ RI signaling.

MAPK signaling is an important therapeutic target for allergic disease (Abdel-Raheem et al., 2005). We identified an important role for Syk, ERK1/2, and p38 in cytokine signaling, but JNK had little to no influence (De Castro et al., 2010). Quercetin inhibited ERK1/2 phosphorylation following antigen stimulation suggesting ERK mediated cytokine and chemokine release occurs. Previous studies suggested that NF- κ B is involved in the expression of an array of inflammatory cytokines and MC adhesion molecules (Marquardt and Walker, 2000). Quercetin inhibited DNP-HSA/IgE induced NF- κ B activation indicating its ability to inhibit inflammatory cytokine and adhesion molecule

expression in MCs. When added alone, quercetin also inhibited the levels of total and phosphorylated Lyn, but had no effects on PLC γ 1. The specific mechanisms by which quercetin inhibits Lyn activity now warrant further investigation.

AC therapeutics can be classified into anti-histamines, histamine receptor antagonist, non-steroidal anti-inflammatory drugs, hormones, and immunosuppressants. However, olopatadine eye-drops as MC stabilizers do not inhibit the release of neutrophils (Uchio et al., 2008). The long-term use of olopatadine eye-drops leads to side effects such as mouth congestion and headache (Maiti et al., 2011; Dakhale et al., 2016). From a critical evaluation of the available literature on the biological effects of quercetin, including data related to safety, it may be concluded that quercetin, at estimated dietary intake levels (daily doses of supplemental quercetin of 200–1200 mg), would not produce adverse health effects (Harwood et al., 2007). In conclusion, we show that quercetin inhibits OVA-induced allergic conjunctivitis in 16 C57BL/6 mice, highlighting its potential as a mast cell stabilizer. Quercetin may be developed a drug candidate for allergic conjunctivitis treatment. Quercetin was found to inhibit DNP-HSA/IgE induced Ca^{2+} influx, MC degranulation, and chemokine release in LAD2 cells. Quercetin also inhibited DNP-HSA/IgE induced Lyn/PLC γ /IP3R- Ca^{2+} and Lyn/ERK1/2, Lyn/NF- κ B activation. Although quercetin treatment did not influence PLC γ 1 phosphorylation, it inhibited total and phosphorylated Lyn levels. In addition, quercetin display inhibitory effects towards Lyn kinase which is similar like its inhibitor. And Lyn inhibitors (Bafetinib, 10 μM) arrest Lyn kinase activity, and quercetin reduce the activation of Lyn kinase under agonist treatment (Tolimidone, 10 μM), so it can be preliminarily determined that quercetin can inhibit allergic conjunctivitis as a Lyn kinase inhibitor. We therefore highlight quercetin as a novel Lyn kinase inhibitor, but the mechanism (s) of this inhibition require further investigation.

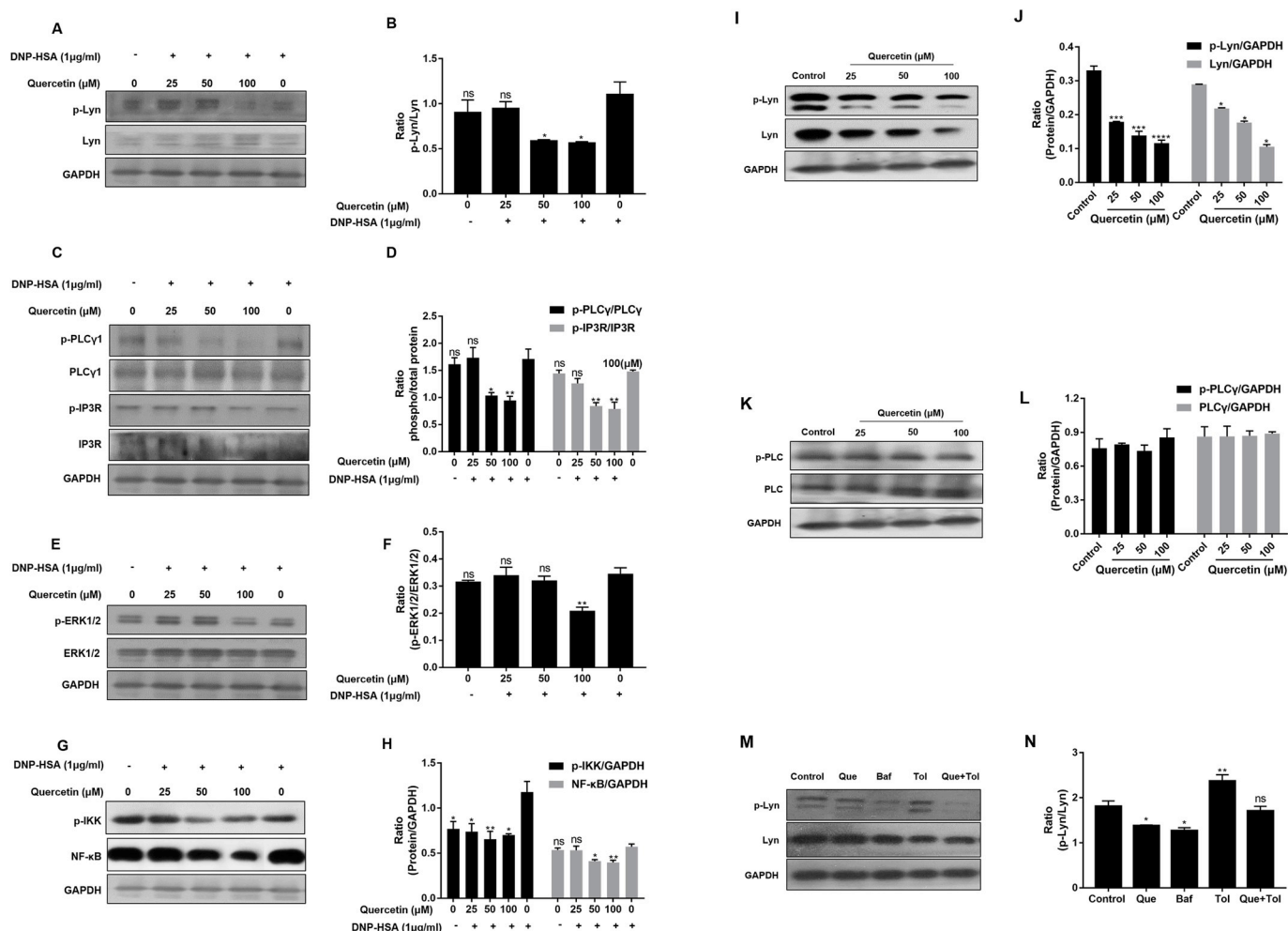


Fig. 8. Quercetin decreased the activation of the Lyn/PLC γ signaling pathway regulating calcium fluctuations in mast cells.

Effect of quercetin on the protein expression of Lyn, PLC γ , IP3R, ERK and NF- κ B in LAD2 cells. (A) Western blot analysis of the expression levels of Lyn, Phosphorylation-Lyn and GAPDH in LAD2 cells treated with quercetin and DNP-HSA. (B) Quantification of Phosphorylation-Lyn protein expression in (A) by densitometric analysis. (C) Western blot analysis of the expression levels of PLC γ , IP3R, Phosphorylation-PLC γ , Phosphorylation-IP3R and GAPDH in LAD2 cells treated with quercetin and DNP-HSA. (D) Quantification of Phosphorylation-PLC γ , Phosphorylation-IP3R protein expression in (C) by densitometric analysis. (E) Western blot analysis of the expression levels of ERK1/2, Phosphorylation-ERK1/2 and GAPDH in LAD2 cells treated with quercetin and DNP-HSA. (F) Quantification of Phosphorylation-ERK1/2 protein expression in (E) by densitometric analysis. (G) Western blot analysis of the expression levels of Phosphorylation-IKK, NF- κ B and GAPDH in LAD2 cells treated with quercetin and DNP-HSA. (H) Quantification of Phosphorylation-IKK and NF- κ B protein expression in (G) by densitometric analysis. (I) Western blot analysis of the expression levels of Lyn, Phosphorylation-Lyn in LAD2 cells treated with quercetin. (J) Quantification of Lyn and Phosphorylation-Lyn protein expression in (I) by densitometric analysis. (K) Western blot analysis of the expression levels of PLC γ and Phosphorylation-PLC γ in LAD2 cells treated with quercetin. (L) Quantification of PLC γ and Phosphorylation-PLC γ protein expression in (K) by densitometric analysis. (M) Western blot analysis of the expression levels of Lyn, Phosphorylation-Lyn in LAD2 cells treated with quercetin (200 μ M), Bafetinib (10 μ M), Tolimidone (10 μ M) and quercetin (200 μ M) combine with Tolimidone (10 μ M). (N) Quantification of Phosphorylation-Lyn protein expression in (M) by densitometric analysis. The data are presented as mean $\pm$ S.D. (n = 5). One-way ANOVA (Bonferroni's multiple comparisons test) was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols for using the mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045). All animals were operated on under Pentobarbital sodium anesthesia.

Author contributions

Yuanyuan Ding wrote the manuscript. Langchong He, Yuanyuan Ding and Tao Zhang contributed to experimental design. Chaomei Li, Yongjing Zhang, Pengyu Ma and Tingting Zhao performed experiments

and analyzed data, together with Delu Che, Jiao Cao, Jue Wang and Rui Liu edited the manuscript.

Declaration of transparency and scientific rigour

This declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2019.110924>.

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