Quercetin Improves Inflammation, Oxidative Stress, and Impaired Wound Healing in Atopic Dermatitis Model of Human Keratinocytes

Burcin Beken, MD,¹ Riza Serttas, PhD,² Mehtap Yazicioglu, MD,¹
Kader Turkecul, PhD,² and Suat Erdogan, PhD²

Background: Atopic dermatitis (AD) is a common inflammatory skin disease with complex pathogenesis. Natural flavonoids exhibit strong anti-inflammatory and antioxidant properties in many human diseases. In this study, the potential bioactive effect of quercetin, a polyphenolic plant-derived flavonoid, on the AD model of human keratinocytes was evaluated.

Methods: Immortalized human HaCaT keratinocytes were treated with interleukin (IL) -4, -13, and tumor necrosis factor- α to mimic AD features *in vitro*. Then effects of quercetin on inflammation, oxidative stress, and wound healing were assessed.

Results: Pretreatment of the cells with 1.5 μ M of quercetin significantly reduced the expression of AD-induced IL-1 β , IL-6, IL-8, and thymic stromal lymphopoietin, while it strongly enhanced the expression of superoxide dismutase-1 (SOD1), SOD2, catalase, glutathione peroxidase, and IL-10. Quercetin promoted wound healing by inducing epithelial–mesenchymal transition, which was supported by the upregulation of Twist and Snail mRNA expression. Unexpectedly, quercetin pretreatment of AD-induced cells upregulated the mRNA expression of occludin and E-cadherin, while downregulating matrix metalloproteinase 1 (MMP1), MMP2, and MMP9 expression. The pretreatment inhibited AD-induced phosphorylation of extracellular signal-regulated kinase 1/2/mitogen-activated protein kinase (ERK1/2 MAPK) and the expression of nuclear factor-kappa B (NF- κ B), but it did not alter signal transducer and activator of transcription 6 (STAT6) phosphorylation.

Conclusion: Quercetin may serve as a potential bioactive substance for atopic dermatitis-related symptoms through anti-inflammatory and antioxidant activities along with its acceleration of wound healing via ERK1/2 MAPK and NF- κ B pathways.

Keywords: atopic dermatitis, inflammation, quercetin, keratinocyte, wound healing

Introduction

ATOPIC DERMATITIS (AD) is the most common, chronic, relapsing inflammatory skin disease in childhood characterized by pruritic and eczematoid skin lesions with typical morphological distribution, affecting up to 10%–20% of children.¹ Epidermis acts as a barrier both with physical and functional properties, and defects of this barrier have been considered to be the initial step of AD development.^{2,3} Keratinocytes are actively involved in cutaneous Type 2 helper T (Th2) cell dominant immune reactions by producing various proinflammatory cytokines and chemokines initiated by inflammatory stimuli, mechanical injury, or activation of Toll-like receptors and protease-activated receptors.^{4,5}

Thymic stromal lymphopoietin is highly expressed in epithelial cells and plays as a key role in early stages of allergic inflammation by activating dendritic cells, which can further promote naive CD4⁺ T cells to differentiate into proinflammatory Th2 cells.⁶ Intense pruritus is a characteristic and often debilitating feature of AD, causing skin damage and further allergen and/or microorganism penetration and also impairing both the patients' and their parents' quality of life.^{7–9} Tissue repair is also impaired in AD, that IL-4 exposure is associated with increased matrix metalloproteinase-9 (MMP-9) and decreased fibronectin.¹⁰

Because of the complexity of the disease and the lack of exact-known underlying mechanisms, it is difficult to develop more-effective management strategies for AD. Flavonoids are a large group of low-molecular-weight

Departments of ¹Pediatric Allergy and Immunology, and ²Medical Biology, School of Medicine, Trakya University, Edirne, Turkey.

secondary plant metabolites known to have a wide variety of effects, including antioxidant, antibacterial, antiviral, anti-inflammatory, anti-angiogenic, and also anti-allergic effects.^{11,12} Quercetin is a polyphenolic flavonoid found in several foods that are consumed in daily diet.¹³ The anti-allergic properties of quercetin, such as the inhibition of histamine release from basophils and mast cells and also inhibition of proinflammatory cytokines¹⁴ and interleukin-4 (IL-4) and IL-13,¹⁵ have been shown by several *in vitro* and *ex vivo* studies. However, there are a limited number of studies exploring the effects of quercetin on AD. Thus, our study aimed to investigate the effects of quercetin on inflammation, oxidative damage, and wound healing in the AD model of HaCaT keratinocytes.

Methods and Materials

Cell culture and reagents

HaCaT immortalized human keratinocytes were provided by Dr. Murat Demirbilek (Hacettepe University, Ankara, Turkey). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM)/Hams' F12 50/50 medium (Wisent, Canada) consisting of 10% fetal bovine serum (Life Technologies) and penicillin/streptomycin. Cells were incubated at 37°C and 5% CO₂. Quercetin, dexamethasone (Sigma-Aldrich Chemie, Taufkirchen, Germany), IL-4 (Thermo Fisher Scientific, Waltham, MA), IL-13 (Sino Biological, China), and TNF- α (GenScript, Piscataway) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at -20°C until use.

Establishing AD-like dermatitis

According to the previous studies investigating the optimal cytokine milieu inducing inflammation and thymic stromal lymphopoietin (TSLP) secretion, TNF- α , IL-4, and IL-13 (called AD-inducing agents) were administered to the culture medium at a concentration of 10, 50, and 50 ng/mL, respectively. Then, the cells were further incubated for 24 h to stimulate atopic-like dermatitis.¹⁶⁻²¹

To determine the effects of quercetin and dexamethasone, HaCaT cells were preincubated with 1.5 μ M quercetin or 1 μ M dexamethasone for 6 h before the AD model was generated.²¹ After 24 h of incubation, culture supernatants were collected and stored at -80°C until assayed.

Determination of cell viability in HaCaT keratinocytes

Keratinocytes were seeded into 96-well plates (10⁴ cells/well) for 16 h, then exposed to quercetin (0.39–50 μ M) or 1 μ M of dexamethasone²² in serum-free medium for 6 h. After treatments, cell survival was determined by the 3-(4,5-dimethylthiazid-2-yl)-2,5 diphenyltetrazolium bromide reduction assay (MTT) (Sigma-Aldrich).²³ The absorbance of the groups was measured on a plate reader with a wavelength of 570 nm (Multiskan GO, Thermo Scientific, Vantaa, Finland). The cell survival was calculated by the following equilibration: OD sample/OD control $\times$ 100.

Examination of apoptotic cell ratio and cell cycle phases

Keratinocytes were plated in 25 cm² flasks overnight, and then the media were removed. After establishing the AD model with the pretreatments with quercetin and dexamethasone, keratinocytes were trypsinized, washed with phosphate-buffered saline (PBS), and resuspended in annexin V binding buffer (ABB). The cells were then washed again, resuspended, and stained with Annexin V (Life Technologies, Carlsbad, CA). The stained cells were then incubated for 15 min at room temperature in the dark. The samples were centrifuged at 300 g for 5 min, resuspended in ABB, and reincubated with propidium iodide solution in the dark. Dead and apoptotic cell ratios were determined by an image-based cytometer (Life Technologies).²⁴

To evaluate the cell phase distribution, keratinocytes were treated as described above, then detached with trypsin, fixed in 70% ethanol, and stored at -20°C overnight. Keratinocytes were then rinsed in PBS, and the cell phases in the population were analyzed by a cytometer using a commercial kit (Life Technologies).²⁴

Evaluation of mRNA expression

Total ribonucleic acid (RNA) was isolated from the cells using a commercial kit. High-purity RNAs were converted to complementary deoxyribonucleic acid (DNA) (Thermo Fisher Scientific). Complementary DNA synthesis was achieved with the high-capacity kit using 500 ng total RNA (Thermo Fisher Scientific). mRNA expression were assessed by real-time polymerase chain reaction (PCR) (Applied Biosystems, Foster City, CA) as described previously.²⁵ The primer sequences used for PCR experiments are presented in Table 1.

Assessment of cell migration

For the migration assay, HaCaT cells were grown to confluence in a 12-well plate and, following 24-h incubation they were scratched using a sterile 0.2 mL pipette tip. Then the plates were rinsed with PBS and refreshed with serum-free medium containing 1.5 μ M quercetin or 1 μ M dexamethasone and AD-inducing agents as indicated above. Cell migration was recorded using an inverted microscope (ZEISS, Axio Vert A.1, Germany) for 48 h. The area of wound closure was calculated using a software.²⁶

Quantification of TSLP concentration

The concentration of TSLP found in cell homogenates was detected by ELISA according to the manufacturer's instructions using a specific ELISA kit (Thermo Fisher Scientific).²⁷ The minimal detection limit for this kit is set to 3 pg/mL. In brief, cells were collected after 24 h incubation, and then the cytosolic homogenates were extracted, pipetted into the plate, and allowed to stand at ambient temperature for 2.5 h. After washing the plates with PBS four times, biotinylated anti-human TSLP antibody was added and incubated for 1 h at room temperature (RT). Then, streptavidin-HRP reagent was added after the plates were washed four times and incubated for 45 min at RT. Subsequently, a TMB substrate solution was added, developed in dark RT for 30 min, and a stop solution was added. Finally, the plate was

TABLE 1. SEQUENCE OF FORWARD AND REVERSE PRIMERS USED IN THE STUDY FOR mRNA AMPLIFICATION

<i>Gene and accession number</i>	<i>Primer sequence</i>
<i>IL-1β</i> NM_000576.2	F: 5'-TGGGTAATTTTGGGATCTACACTCT-3' R: 5'-AATCTGTACCTGTCTGCGTGT-3'
<i>IL-6</i> XM_005249745.5	F: 5'-GCCTTCGGTCCAGTTGCCTT-3' R: 5'-GCAGAATGAGATGAGTTGTC-3'
<i>IL-8</i> NM_001354840.1	F: 5'-GGAAGGAACCATCTCACTGT-3' R: 5'-CAGTGTGGTCCACTCTCAATC-3'
<i>IL-10</i> NM_000572.2	F: 5'-GCCTAACATGCTTCGAGATC-3' R: 5'-CTCATGGCTTTGTAGATGCC-3'
<i>Catalase</i> NM_001752.3	F: 5'-CCAACAGCTTTGGTGTCTCCG-3' R: 5'-GGCCGGCAATGTTCTCACAC-3'
<i>SOD1</i> NM_000454.4	F: 5'-ACGGTGGGCCAAAGGATGAA-3' R: 5'-TCATGGACCACCAGTGTGCG-3'
<i>SOD2</i> NM_001322820.1	F: 5'-AGAAGCACAGCCTCCCCGAC-3' R: 5'-GGCCAACGCCTCCTGGTACT-3'
<i>GPx</i> NM_001329503.1	F: 5'-TCGGTGTATGCCTTCTCGGC-3' R: 5'-CCGCTGCAGCTCGTTCATCT-3'
<i>Snail</i> NM_005985.3	F: 5'-CAACCCACTCAGATGTCAA-3' R: 5'-CATAGTTAGTCACACCTCGT-3'
<i>Twist</i> NM_000474.3	F: 5'-GGGAGTCCGCAGTCTTAC-3' R: 5'-CCTGTCTCGCTTTCTCTTT-3'
<i>Vimentin</i> XM_006717500	F: 5'-AATGACCGCTTCGCCAAC-3' R: 5'-CCGCATCTCCTCCTCGTAG-3'
<i>E-cadherin</i> NM_001317186.	F: 5'-TTGACGCCGAGAGCTACAC-3' R: 5'-GTCGACCGGTGCAATCTT-3'
<i>Occludin</i> NM_001205254.1	F: 5'-TGCATGTTTCGACCAATGC-3' R: 5'-AAGCCACTTCCTCCATAAGG-3'
<i>MMP1</i> NM_001145938.1	F: 5'-ACAGCCCAGTACTTATTCCTTTG-3' R: 5'-GGGCTTGAAGCTGTTACGA-3'
<i>MMP2</i> NM_001302510.1	F: 5'-TCTCCTGACATTGACCTTGGC-3' R: 5'-CAAGGTGCTGGCTGAGTAGATC-3'
<i>MMP9</i> NM_004994.2	F: 5'-CCTTGTGCTCTTCCCTGGAG-3' R: 5'-GGCCCCAGAGATTTGACTC-3'
<i>GAPDH</i> NM_001289745.2	F: 5'-TTGGTATCGTGGAAGGACTCA-3' R: 5'-TGTCATCATATTGGCAGGTTT-3'

F, forward; GAPDH, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; IL, interleukin; MMP, matrix metalloproteinase; R, reverse; SOD, superoxide dismutase.

read in the microplate reader, with absorbance at 450 nm within 5 min. TSLP analysis for all samples was performed at the same time, and twice, according to the manufacturer's instructions.

Western blot analysis

HaCaT cells were pretreated with 1.5 μ M quercetin or 1 μ M dexamethasone for 6 h and then exposed to AD-inducing agents for a further 24 h. Seventy-five μ g of protein samples from each group were separated on polyacrylamide gel and transferred onto a PVDF membrane (Life Technologies). Following blocking, the membranes were treated with primary antibodies against phospho-signal transducer and activator of transcription 6 (STAT6) and phospho-extracellular signal-regulated kinase 1/2 (p-ERK1/2) (Thermo Fischer Scientific, Waltham, MA), nuclear factor-kappa B (NF- κ B), and β -actin (Novus Biologicals, LLC) overnight at 4°C. The membrane was then rinsed in TBS and incubated with the anti-rabbit/anti-mouse IgG using a chemiluminescence western blot substrate kit (Thermo Fisher Scientific) for 2 h at RT. The densities of the bands on the gel were analyzed using the imaging system, corrected against B-actin as the loading control (Bio-Rad ChemiDoc MP System, Carlsbad, CA).²⁸

Data analysis

Results are presented as mean $\pm$ standard deviation. The differences between the groups were analyzed by ANOVA using SPSS (v19) followed by Duncan's multiple range test for multiple comparisons. The *P*-values less than 0.05 were considered as significant.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Results

Quercetin does not adversely affect cell survival and cell cycle phases

Quercetin (Fig. 1A) had no cytotoxic effect up to a concentration of 25 μ M (Fig. 1B). We chose to use quercetin's non-toxic medium concentration of 1.5 μ M in future experiments. Image-based cytometry analysis and the MTT test showed that application of IL-4, IL-13, and TNF- α did not alter the percentages of viable or apoptotic cells (Fig. 1C). A similar treatment of the cells as described above did not produce a significant difference in cell cycle distribution (Fig. 1D).

FIG. 1. Quercetin treatment does not alter cell viability and cell cycle progress. The chemical structure of quercetin (A). HaCaT cells were treated with 0.39–50 μM of quercetin for 24 h, and cell viability was determined by MTT assay (B). * $P < 0.05$ versus untreated. Cells were pretreated with 1.5 μM quercetin or 1 μM dexamethasone for 6 h and then exposed to 50 ng/mL IL-4, 50 ng/mL IL-13, and 10 ng/mL TNF- α for further 24 h; then the rates of death and apoptotic cells (C) and cell cycle phases were determined by image-based cytometry (D). Untreated, control; AD, atopic dermatitis; Quer, quercetin; Dexa, dexamethasone.

Quercetin regulates expression of inflammatory mediators

The increased expression of IL-1 β , IL-6, and IL-8 in HaCaT keratinocytes treated with AD-inducing agents were significantly downregulated by quercetin pretreatment (Fig. 2A–C). Although AD-inducing agents significantly reduced the mRNA expression of anti-inflammatory cytokine IL-10, quercetin pretreatment upregulated its expression up to 100-fold (Fig. 2D). On the contrary, dexamethasone was more effective in downregulating IL-1 β expression in AD cells than in quercetin, but was less effective in the expression of IL-6, IL-8, and IL-10.

Quercetin supports the antioxidant defense system

The exposure of HaCaT cells to IL-4, IL-13, and TNF- α significantly downregulated the expression of superoxide dismutase-1 (SOD1), SOD2, catalase (CAT), and glutathione peroxidase (GPx) (Fig. 3A–D). Conversely, pretreatment of the cells with 1.5 μM quercetin significantly induced expression of indicated antioxidant enzymes. The upregulating effect of quercetin was similar for CAT and GPx, better in SOD2, and weaker for SOD1 compared to dexamethasone treatment (Fig. 3A–D).

Quercetin promotes wound repair

We performed a cell migration assay to investigate the effect of quercetin on tissue repair. AD-stimulating agents

significantly delayed wound closure compared to the control group, indicating that tissue repair is impaired in AD (Fig. 4A, B). On the contrary, the single application of quercetin or its coadministration with AD-inducing agents was significantly effective in closing the wound gap (Fig. 4A, B). It was seen that dexamethasone was less effective than quercetin on wound closure (Fig. 4A). The mRNA expression of Twist was upregulated by 50% (Fig. 4C), while Snail expression was downregulated by 70% (Fig. 4D) in the AD model. However, pretreatment of the cells with 1.5 μM quercetin upregulated the mRNA expression of Twist and Snail up to 3 and 10 fold, respectively (Fig. 4C, D). The dexamethasone activity on these two transcription factors was also lower than that of quercetin (Fig. 4C, D).

The AD-inducing agents significantly downregulated the mRNA expression of the epithelial junctional proteins, E-cadherin (Fig. 4E), and occludin (Fig. 4F), while quercetin upregulated these proteins by 6.5 and 4.2 fold, respectively, compared to the untreated cells. Quercetin pretreatment on AD model of keratinocytes significantly increased E-cadherin and occludin expression compared to the AD group. This effect was lower in dexamethasone group (4E–F). While AD-inducing agents did not alter vimentin expression, quercetin increased it by 40%; however, pretreatment of AD model of keratinocytes with quercetin or dexamethasone had a minimal increasing effect. (Fig. 4G).

We also examined the effect of quercetin on MMP expression in AD-developed cells. Induction of AD upregulated the expression of MMP1, MMP2, and MMP9 by 2.2,

FIG. 2. Quercetin downregulates AD-induced cytokine mRNA expression. HaCaT cells were pretreated with 1.5 μ M quercetin or 1 μ M dexamethasone for 6 h and then exposed to 50 ng/mL IL-4, 50 ng/mL IL-13, and 10 ng/mL TNF- α for a further 24 h. Then mRNA expression of IL-1 β (A), IL-8 (B), IL-6 (C), and IL-10 (D) were evaluated by real-time qPCR. Untreated, control; AD, atopic dermatitis; Quer, quercetin; Dexa, dexamethasone. * P < 0.05 versus untreated, ** P < 0.05 versus AD.

FIG. 3. Quercetin application induces AD-suppressed antioxidant enzymes' mRNA expression. HaCaT cells were pretreated, and AD was induced, presented as before. Expression of SOD1 (A), SOD2 (B), catalase (C), and GPx (D) were assessed by real-time qPCR. Untreated, control; AD, atopic dermatitis; Quer, quercetin; Dexa, dexamethasone. * P < 0.05 versus untreated, ** P < 0.05 versus AD.

FIG. 4. Quercetin supports wound repair disrupted by AD-inducing agents and modulates EMT-related gene expression. HaCaT cells were scratched using a sterile 0.2 mL pipette tip, following 6 h of pretreatment with quercetin or dexamethasone; AD-inducing agents were added to the culture media and allowed to incubate further 48 h. Incubation was performed in serum-free medium to prevent cell proliferation. The wound width was measured under a microscope using software at the beginning of incubation, 24 and 48 h (A, B). Then, mRNA expression of Twist (C), Snail (D), E-cadherin (E), occludin (F), and vimentin (G) was determined by real-time qPCR. Untreated, control; AD, atopic dermatitis; Quer, quercetin; Dexa, dexamethasone. * $P < 0.05$ versus untreated, ** $P < 0.05$ versus AD. EMT, epithelial mesenchymal transition.

1.6, and 3.1 folds, respectively, in HaCaT cells (Fig. 5A–C). The application of quercetin alone or both with AD agents significantly downregulated the mRNA expression of MMPs. The downregulation effect of quercetin on MMP1 and MMP9 expression was stronger than dexamethasone (Fig. 5A–C).

Quercetin inhibits TSLP secretion in AD model of HaCaT keratinocytes

We showed that the protein concentration of TSLP in cell lysates was increased approximately fivefold by AD-inducing agents *in vitro* (Fig. 5D). While quercetin pretreatment did not alter TSLP release, it decreased the level in AD-induced cells close to basal level. Dexamethasone was less effective than quercetin in inhibiting TSLP secretion (Fig. 5D).

Quercetin inhibits AD-induced ERK1/2 phosphorylation and NF- κ B expression

Exposure of the cells to AD-inducing agents caused the phosphorylation of ERK 1/2 and STAT6, and upregulated the expression of NF- κ B protein (Fig. 6A). Pretreatment of AD-stimulated HaCaT cells with quercetin significantly reduced the phosphorylation of ERK 1/2 and decreased the protein expression of NF- κ B, but it was ineffective on AD-induced STAT6 phosphorylation (Fig. 6A–E). The effect of dexamethasone treatment on NF- κ B expression, and

on STAT6 and ERK 1/2 phosphorylation was almost identical to quercetin (Fig. 6A–E).

Discussion

Flavonoids are a large group of plant-derived metabolites that have been shown to exhibit anti-inflammatory and antioxidant properties.^{15,27,29} We showed that a low concentration of quercetin had strong anti-inflammatory activity through downregulating the mRNA expression of IL-1 β , IL-6, and IL-8 and upregulating the expression of IL-10 without adversely affecting cell survival in AD model of HaCaT keratinocytes. It has been assumed that concentration of quercetin 0–10 μ M is accepted as low, whereas midranges are 10–200 μ M from the studies presented.³⁰ Lower concentrations appear to be achievable by diet, while the higher concentrations might require supplementation. Quercetin has been reported to inhibit IL-1 β and IL-6 secretion and induce IL-10 expression in lipopolysaccharide-induced inflammation in mice³¹ and RAW264 macrophages.³² Moreover, a significant reduction both in AD severity and eosinophil numbers after 2 months of a flavonoid-rich vegetarian diet was reported.³³ In addition to the previous studies showing the inhibitory effect of quercetin on indicated inflammatory cytokines, our study has demonstrated that quercetin has a stronger anti-inflammatory effect compared to dexamethasone.

Oral administration of quercetin has been shown to decrease inflammation in a 2,4-dinitrochlorobenzene-induced

FIG. 5. Quercetin regulates the expression of MMPs and the TSLP level. HaCaT cells were pretreated with 1.5 μ M quercetin or 1 μ M dexamethasone for 6 h and then exposed to 50 ng/mL IL-4, 50 ng/mL IL-13, and 10 ng/mL TNF- α for a further 24 h. Expression of MMP-1 (A), MMP-2 (B), and MMP-9 (C) was evaluated by RT-qPCR. TSLP concentration found in the culture supernatant was determined by ELISA method (D). * $P < 0.05$ versus untreated, ** $P < 0.05$ versus AD. TSLP, thymic stromal lymphopoietin; MMP, matrix metalloproteinase.

FIG. 6. Modulation of STAT6, p-ERK, and NF- κ B protein expression by quercetin. HaCaT cells were pretreated with 1.5 μ M quercetin or 1 μ M dexamethasone for 6 h and exposed to 50 ng/mL IL-4, 50 ng/mL IL-13, and 10 ng/mL TNF- α for 24 h. Then the protein expression of p-STAT6, p-ERK1/2, and NF- κ B was evaluated by western blot analysis (A–E). Intensities of immunoreactive bands were quantified by densitometric analysis, and signal intensity was normalized to that of β -actin as the loading control. Values are expressed as a ratio of the untreated control. UT, untreated; AD, atopic dermatitis; Quer, quercetin; Dexa, dexamethasone. * P < 0.05 versus untreated, ** P < 0.05 versus AD.

AD mouse model.³⁴ Oral intake of astragalin (persimmon leaf-derived flavonoid) also found to be effective in ameliorating AD severity and decreasing transepidermal water loss with no apparent side effect.³⁵ However, low oral bioavailability due to their poor aqueous solubility has been a major concern for the majority of flavonoids. To address this problem, numerous promising strategies, such as using an absorption enhancer, structural transformation, and pharmaceutical technologies such as carrier complexes or

nanotechnology have been developed.³⁶ Topical or oral quercetin, especially the highly bioavailable formulations can be tried in AD patients in future studies.

Pretreatment with quercetin also inhibited the TSLP secretion in present experimental AD model. In a previous study, quercetin was found to be effective in inhibiting the production of TSLP in allergic airway mouse model.²⁷ Similar to our study, Jung et al. demonstrated an inhibitory effect of quercetin on TSLP secretion in an AD-like Nc/

Nga mouse model, which is enhanced by tannic acid.³⁷ The downregulation of P38, JNK, NF- κ B, and ERK phosphorylation have been shown to reduce TSLP, IL-6, TNF- α , and IL-1 β expression.^{38,39} Thus, MAPK and NF- κ B downregulation by quercetin administration may play a role in reducing TSLP production.

Recent evidence shows that oxidative stress caused by reactive oxygen and nitrogen species plays a role in the development of allergic and inflammatory skin diseases.⁴⁰ Reactive oxygen species (ROS) can act as second messenger molecules, activating immune cells as well as nonimmune cells such as keratinocytes to release cytokines and chemokines via activation of transcription factors and signaling pathways, such as NF- κ B and p38 MAPK.⁴¹ A previous study by Omata et al. also suggested that increased oxidative stress is involved in the pathophysiology of childhood AD by demonstrating impaired homeostasis of oxygen/nitrogen radicals and increased urinary 8-hydroxy-29-deoxyguanosine.⁴² In the present study, although we did not measure ROS levels in keratinocytes, decreased expression of catalase, GPx, SOD1, and SOD2 after treating the HaCaT cells with IL-4, IL-13, and TNF- α may indicate that AD leads to oxidative stress. However, pretreatment with quercetin was able to restore the expression of these antioxidant enzymes.

Hamalainen et al. studied the anti-inflammatory effects of eight flavonoids including quercetin, and showed that they all inhibited the activation of nuclear factor- κ B (NF- κ B), which is a significant transcription factor for iNOS.⁴³ It was also shown that IL-1 β and TNF- α -mediated TSLP gene expression in human airway epithelial cells was mediated by NF- κ B.³⁹ We conclude that the anti-inflammatory effect of quercetin in the present study was based on its antioxidant and TSLP downregulating properties via ERK1/2 and NF- κ B pathways but not STAT6. The fact that cytokine-driven STAT6 regulation has been implicated in diseases that natural polyphenols may influence. Cortes and coworkers⁴⁴ showed that among the flavonols and flavonoids fisetin, apigenin, and luteolin clearly inhibit IL-4-induced STAT6 phosphorylation. In contrast, flavonol and myricetin have little effect on the activation of STAT6 in mixed lymphocyte culture. Variable effect of similar flavonoids at the same concentration (40 μ M) on STAT6 phosphorylation may possibly be related to their structural changes.⁴⁴ Quercetin at concentrations of 4 μ M or higher suppresses STAT6 phosphorylation that was increased by IL4 in human nasal epithelial cells.⁴⁵ The low dose of quercetin used in our study might be the reason for its ineffectiveness on stat 6 phosphorylation.

Wound healing is a natural restorative response that involves cell migration in tissue damage. The potential of quercetin in treating AD has yet to be sufficiently studied. We have found that quercetin improves wound closure impaired by AD even better than dexamethasone. According to previous data, quercetin can accelerate wound healing by downregulating proinflammatory cytokines and supporting antioxidant capacity.⁴⁶ In the present study, administration of quercetin significantly upregulated the expression of transcription factors regulating epithelial–mesenchymal transition (EMT) such as Snail and Twist, together with E-cadherin and occludin. A slight increase in vimentin expression was also observed. The classic description of EMT as the transformation of epithelial cells into mesenchymal cells⁴⁷ has been changed from “transformation” to “tran-

sition” because of the process not being a shift between two states, epithelial or mesenchymal. Recent studies have pointed to a greater flexibility in this transitional process and showed that cells do not only exhibit full epithelial and full mesenchymal states but also intermediary phases called partial EMT.⁴⁸ A partial EMT state has been demonstrated in many developmental, wound healing, fibrosis, and cancer processes through the existence of both epithelial and mesenchymal characteristics as we found here.

Keratinocytes express various proteins, including MMPs, which enable extracellular matrix degradation to facilitate cell migration. Therefore, the activity of MMPs normally increases in healthy wound healing, but overproduction of these enzymes disrupts normal tissue repair by destroying growth factors, extracellular matrix, or removing cell surface receptors.⁴⁹ The effect of flavonoids on cell migration and wound healing depends on cell types and concentration. For example, 20–100 μ M quercetin inhibits EMT processes in TGF- β 1-activated human colorectal cancer cells,⁵⁰ prostate cancer stem cells,²⁸ or in hepatocellular carcinoma through downregulating the expression of p-Akt1, MMP2, and MMP9.⁵¹ It has been shown that quercetin reduces the formation of the extracellular matrix without significantly impaired fibroblast cell growth, which is ideal for diminishing scar formation.⁵² Although there is no extracellular matrix in our experimental model, suppression of AD-upregulated MMP1, -2, and -9 together with the anti-inflammatory and antioxidant effects of quercetin observed may contribute to wound healing as demonstrated by previous studies.⁴⁶

In conclusion, these results suggest that quercetin has a bioactive effect on inflammation, oxidative stress, and wound healing in AD model of keratinocytes. Quercetin inhibits the crucial cytokines for AD via NF- κ B and ERK1/2 MAPK pathways in HaCaT cells. We provide evidence that quercetin may be a potential therapeutic candidate for AD. Taken into account that the nature of our study being *in vitro*, the results should be supported by further *in vivo* studies.

Acknowledgments

We thank Dr. Murat Demirbilek from Hacettepe University, Ankara, Turkey for his generous cooperation in providing HaCaT keratinocytes. Also, we thank Jay Lewis Allchin for his assistance in the editing of the article.

Author Disclosure Statement

All the authors declare that they had no personal and financial support or commercial associations that might create a conflict of interest in connection with the article.

Funding Information

This study was funded by Trakya University Scientific Research Projects Unit (TUBAP) (Project number: 2019-89).

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Address correspondence to:

*Burcin Beken, MD
Department of Pediatric Allergy and Immunology
School of Medicine
Trakya University
Edirne 22030
Turkey*

E-mail: burcinbeken@gmail.com

Received for publication December 23, 2019; accepted after revision April 15, 2020.