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ORIGINAL RESEARCH

The Effect of Osthole on Transient Receptor Potential Channels: A Possible Alternative Therapy for Atopic Dermatitis

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Introduction: Chronic recurrent skin inflammation and severe itching in patients with atopic dermatitis (AD) significantly impair their quality of life. The H4 histamine receptor plays a key role in histamine-induced itching. During the skin inflammation associated with AD, pro-inflammatory mediators (interleukins, cytokines) are released from neurons. Ultimately, a cascade of reactions leads to the activation and sensitization of transient receptor potential channels (TRP), which exacerbate the inflammation and itching associated with AD. Osthole (OST) is a natural coumarin with a proven versatile pharmacological effect: anti-cancer, anti-inflammatory and immunomodulatory. However, the molecular mechanism of OST in relieving inflammation in histamine-mediated itching is not yet clear.

Purpose: In the studies presented, the possible effect of the OST action on the inhibition of the gene expression of the histamine H4 receptor and the key genes of the TRP channels as well as on the concentration of proinflammatory interleukins was analyzed.

Methods: Inflammation was induced in a 3D skin model and a keratinocyte cell line Normal Human Epidermal Keratinocytes (NHEK) identical to that of AD, and then OST was administered at various doses. The concentrations of IL-4/-13 were determined by ELISA. RNA was isolated from the 3D skin cells and the NHEK cell line, and the qPCR method was used to determine the expression of: IL-4α, H4R, TRPV1, TRPV4, TRPM8 analyzed.

Results: The study showed that OST significantly reduced the secretion of IL-4/-13 in a keratinocyte cell line and in a 3D skin model. In addition, OST was found to significantly decrease the gene expression of IL-4α, H4R, TRPV1, TRPV4 and increase TRPM8 in both the NHEK cell line and the organotypic 3D skin model.

Conclusion: The data obtained provide the first in vitro evidence of itch relief following the application of OST to atopic skin. Research on the use of OST as an active component of emollients in the treatment of AD should be continued in the future.

Keywords: pro-inflammatory cytokines, keratinocytes, clobetasol propionate, CP, TRPV channels, 3D skin

Introduction

Persistent recurrent skin inflammation and severe itching are characteristic of patients with atopic dermatitis (AD).[1–3] The molecular mechanism is not clear enough, even in patients for whom treatment brings relief.

The fluctuating and unpredictable course of AD disease and the relatively limited number of approved drugs with an acceptable risk–benefit ratio continue to pose a major challenge in the treatment of the disease. As our understanding of AD disease evolves with increasing data on phenotypes, biomarkers and etiopathogenesis, we aim to leverage this knowledge to provide personalized treatment with existing and new therapies.[4,5]

AD disease is not a disease that affects only one system. Patients with concurrent atopic comorbidities such as asthma may benefit from treatment with dupilumab (approved for the treatment of severe asthma), and patients with concurrent rheumatoid arthritis, spondyloarthropathies, alopecia areata or vitiligo may benefit most from JAK inhibition. The potential benefit of systemic treatment for other comorbidities such as neuropsychiatric complications known to be associated

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with AD disease needs to be further explored. Eczema that occurs in specific and high-stress sites, such as hand eczema and head and neck eczema, also requires further attention as there are relatively few studies addressing this focus.[5]

Histamine, is a mediator in a variety of conditions, including hives, insect bite reactions, abnormal accumulation of MC's (mast cells) in the tissue, and drug spots.[6] Histamine-binding receptors are a family of G protein-coupled receptors (GPCRs). To date, four subtypes of histamine receptors (H1-H4) have been identified.[7] Studies have shown that the H4 receptor is the most important histamine receptor responsible for the development of itch in AD.[8] In addition, the skin, is known to be an important site for immune inflammation as it is composed of different cell types that respond to bacterial pathogens [bacteria release endotoxins such as lipopolysaccharide (LPS)] and therefore trigger inflammatory responses when they encounter each other.[9]

In cutaneous dermatoses, the factors that trigger itching cause somatosensory neurons to open TRP channels, which then depolarize nerve endings.[10] A signaling cascade is triggered. This triggers action potentials, the neurons then release inflammatory mediators [interleukins IL; chemokines (ChK), proteins; etc.] and send specific signals to the corresponding itch neurons in the spinal cord. This pro-inflammatory response triggers a TRP (Transient Receptor Potential) channel, which leads to an exacerbation of inflammation and itching in AD.

In neurogenic dermatitis, which occurs in chronic pruritus and dermatosis, somatosensory afferents are triggered by factors secreted by different cell types in the skin. They cause permanent itching and skin irritation.[11] In addition, keratinocytes in the periphery release inflammatory mediators and further promote itch.[12,13] The cascades in skin dermatoses described above lead to systemic and neuropathic conditions.[10] If we understand the basic molecular mechanisms, we can carefully analyze the transition to a chronic state in skin dermatoses and respond appropriately, also thanks to an innovative treatment model.

Recent scientific data show that inflammatory mediators can cause increased activity and expression of TRP channels. It has been shown that cytokines from the TH1 group trigger the development of the chronic phase in dermatoses and thereby increase the activity of TRP channels. However, their role in this context has been underestimated. It is hypothesised that selected ChKs that are directly associated with AD induction also enhance TRP channel function in different ways. For example, Th2 cytokines increase TRP channel synthesis and TRP channel transcription, leading to faster channel modulation and sensitization.[14] In addition, they can regulate the action of the selection peptide that triggers itching, thus modulate the function of the epidermal TRP channels.[15] Furthermore, the modulation of the TRP channel and its expression is enhanced by the interaction between different Th2 and their cognate IL's.[16] The development of pruritus and other dermatoses is caused by the release of neurogenic inflammatory mediators and neuropeptides induced by the stimulation of Transient Receptor Potential Vanilloid type 1 (TRPV1).[17,18] Histamine acts as a mediator of TRPV1 by activating the PLA2/LO pathway, resulting in stimulation of sensory neurons, which are the direct cause of chronic itch.[19] At this point, it should be added that TRPV1-/- mice show a reduced need to scratch after trypsin injection, which clearly indicates the key role of TRPV1 in histamine and other inflammatory factor dependence, which should be verified in relation to dermatoses - which we aimed to investigate and demonstrate in the present work.[20] On the other hand, Transient Receptor Potential Vanilloid 4 (TRPV4) plays a specific role in keratinocytes, for example in formation of the skin barrier.[21,22] It is very important that TRPV4 binds directly to β-catenin, leading to maturation of the barrier, which relies on intercellular connections. The β-catenin is a component of the adhesion complex and is responsible for establishing connections between cells.[21] Transient Receptor Potential Melastatin Subfamily 8 (TRPM8; also called cold and menthol receptor 1 or CMR1) is a thermal ion channel receptor. The increased activity of TRPM8 has antipruritic properties that are the result of multistep complex and complementary mechanisms.[23] Considering all available and recent scientific data, we hypothesised that the combination of ChKs of the Th2 subfamily with sensory transducers plays an important role in the severity of disease symptoms. Therefore, this work will help us to understand the role of inflammatory mediators in combination with the expression of TRP channels. This will allow us to understand the mechanisms of interaction between the molecular aspects of TRP channels and the inflammatory mediators that occur mainly in allergy and immune inflammation. In addition, the role of OST in inhibiting the expression of receptors and the secretion of inflammatory mediators will be investigated, which may lead to the development of new therapeutic interventions that can be used in dermatoses in the future. Osthole (OST) (7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one) is a natural coumarin and a major chemical constituent of the plant

Cnidium monnieri (L) Cusson and is considered a promising natural product that could be used pharmacologically in the future. These include anti-cancer[24–26] anti-inflammatory,[27–30] antioxidant,[31] anti-angiogenic,[32] anti-allergic[27,33,34] immunomodulatory[33,35] and hepatoprotective[36–38] activities. In our previous publications, we found that OST is immunosuppressive in peripheral blood mononuclear cells (PBMCs) isolated from children with allergies or asthma[27,30,39–43] and autism spectrum disorders (ASD), in keratinocytes and fibroblast cell lines[28,] and in a 3D skin model.[29] However, the adequate role of OST in histamine-dependent pruritus is not clear, and the molecular mechanisms of its action have not been thoroughly investigated.

Based on published studies, we used histamine and LPS to induce inflammation in human keratinocytes and a 3D skin model and correlated the results with the response of TRP channels to inflammatory agents. To compare the effect of OST as an immunosuppressant, cells were also stimulated with clobetasol propionate (CP), a potent corticosteroid (TCS), a drug used to treat dermatoses.[44,45] This was our positive control to which we compared the results obtained with the OST.

OST as an active substance can modulate the response of the immune system in allergic reactions. To determine whether OST reduces inflammation, its effect on the secretion of the two main anti-inflammatory cytokines IL-4 and IL-13 and on the alteration of gene expression was investigated: IL-4 alpha receptor, histamine receptor 4, TRPV1, TRPV2 and TRPM8 were analyzed in the NHEK cell line and the organotypic 3D skin model treated with histamine and LPS.

Materials and Methods

Chemicals

CP (CAS 25122-46-7), histamine (CAS 51-45-6), LPS from Escherichia coli O111:B4 (EC 297-473-0), and OST (CAS 484-12-8) were obtained from Sigma Aldrich (St. Louis, MO, USA, cat. no. Y0000559, Y0001779, L4391, and Y0001207, respectively). CP (25 mg/mL) was dissolved in DMSO (Sigma Aldrich, St. Louis, MO, USA, cat. no. D8418), histamine (1 mg/mL) and LPS (0.8 mg/mL) were dissolved in double-distilled water, whereas OST (10 mg/mL) was dissolved in 96% ethanol (Chempur, Piekary Śląskie, Poland, cat. no. 653964200). All solutions were filtered through 0.22-µm pore filters, aliquoted, and stored at −20°C for future analysis.

Cell Culture

Cell cultures were established as described by Kordulewska et al 2021.[29] In brief, the Normal Human Epidermal Keratinocyte (NHEK) cell line was purchased from PromoCell GmbH (Heidelberg, Germany, cat. no. C-12005) and cultured in T-75 flasks in keratinocyte medium (Keratinocyte Growth Medium 2 ready to use, PromoCell, Heidelberg, Germany, cat. No. C-20011). Media and supplements were mixed according to the manufacturer's instructions. NHEK cells were incubated at 37°C in a 95% humidified atmosphere with 5% CO2. The culture medium was changed every 2–3 days. NHEK cells were passaged when confluence reached approximately 80%. Only the early passages (3–7) were used for further analysis.

Normal Human Dermal Fibroblasts (NHDF; from juvenile foreskin) were purchased from PromoCell GmbH (Heidelberg, Germany, cat. no. C-12300) and cultured in a T-75 flask in recommended culture medium (PromoCell GmbH, Heidelberg, Germany, cat. no. C-23010). The medium and supplements were mixed according to the manufacturer's instructions and contained 1% penicillin/streptomycin (Sigma Aldrich, St. Louis, MO, USA, cat. no. P4333). The NHDF cell line was incubated at 37°C in a 95% humidified atmosphere and 5% CO2. The culture medium was changed every 2–3 days and the cells were passaged when confluence reached approximately 80–90%.[29]

Organotypic 3D Skin Cultures

The organotypic 3D skin in vitro model was prepared as previously described Kordulewska et al, 2021.[29] The process for developing the 3D skin is shown in Figure 1. The first step was to prepare the collagen/PHF beds. For this purpose, rat tail collagen (Sigma Aldrich, St. Louis, MO, USA, Cat. No. 08-115) was added to a 15 mL conical tube. Then 1.6 mL of reconstitution buffer (1.1% NaHCO3, 0.025N NaOH, 100 mM HEPES, 5X DMEM/F12) was added and mixed well to avoid bubble formation in the collagen. Then 400 µL of NHEK (4x106 cells/mL cell suspension) was added and mixed. Then 400 µL of the collagen/NHEK mixture was aliquoted directly into the center of each MilliCell insert (Millipore, Cat. No.

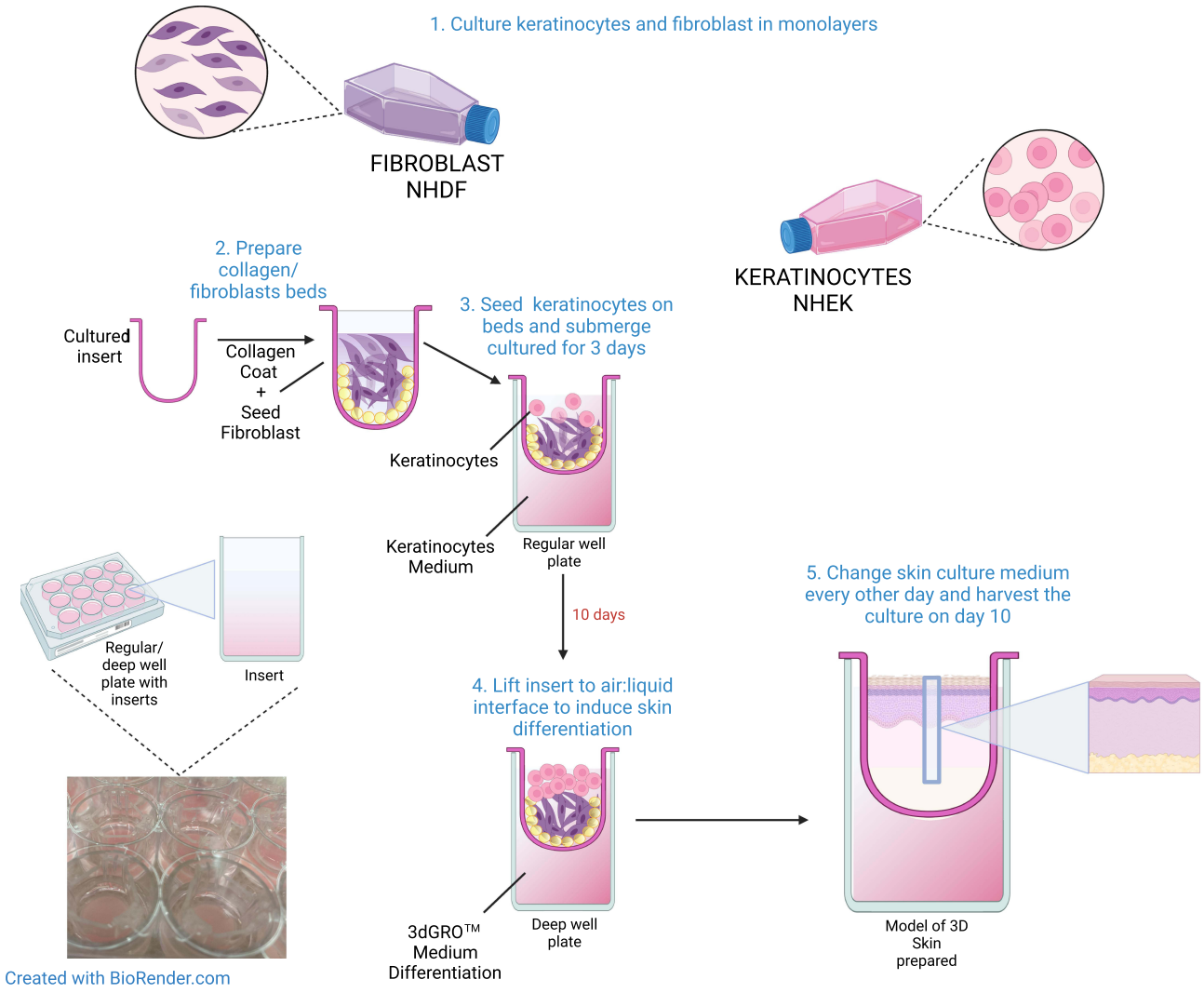

Figure 1 Overview of the preparation of organotypic 3D human skin model. Created using BioRender.com.

MCHT12H48). The inserts/plates were incubated at 37 °C for at least 30 minutes to allow complete polymerization. After this time, 0.5 mL of NHEK cells (4x105 cells/mL cell suspension) were added to each collagen/NHEK bed. 2 mL of the keratinocyte medium was added to the outside of the inserts in a normal 12-well plate. The cells with the inserts were incubated overnight at 37°C. The next day, without removing the original medium, a further 0.5 mL of keratinocyte medium was added to the inside of each insert and incubated at 37°C for a further 2 days. The NHEK were maintained in a submerged culture on the collagen beds for a total of three days. On day 4, the medium was carefully aspirated from each insert. The inserts were then transferred to a 12-well plate (Greiner Bio-One, Cat. No. 665110) with 4.5 mL of 3dGRO™ Skin Differentiation Medium (Sigma Aldrich, St. Louis, MO, USA, Cat. No. SCM310) in each well. The skin culture was incubated at 37°C for a further 10 days. After 10 days of 3D skin culture, approximately 8 to 10 layers of living epithelium had formed.

Organotypic 3D Skin Cells - Incubation with Investigated Substances

Organotypic 3D skin cells were prepared according to the modified protocol described previously. The experimental setup with detailed graphical representations of the performed analyses is shown in Figure 2. Briefly, 3D skin cells were seeded on transfer insert plates (Millipore, cat. no. MCHT12H48) and cultured for 11 days until the cells were fully differentiated. The culture medium was changed every 2 days. After replacing the media inserts with 3D skin, cells were transferred to new deep-well plates. 2 µg/mL LPS or 10^{-3} M histamine was added to the basolateral side, and after 3

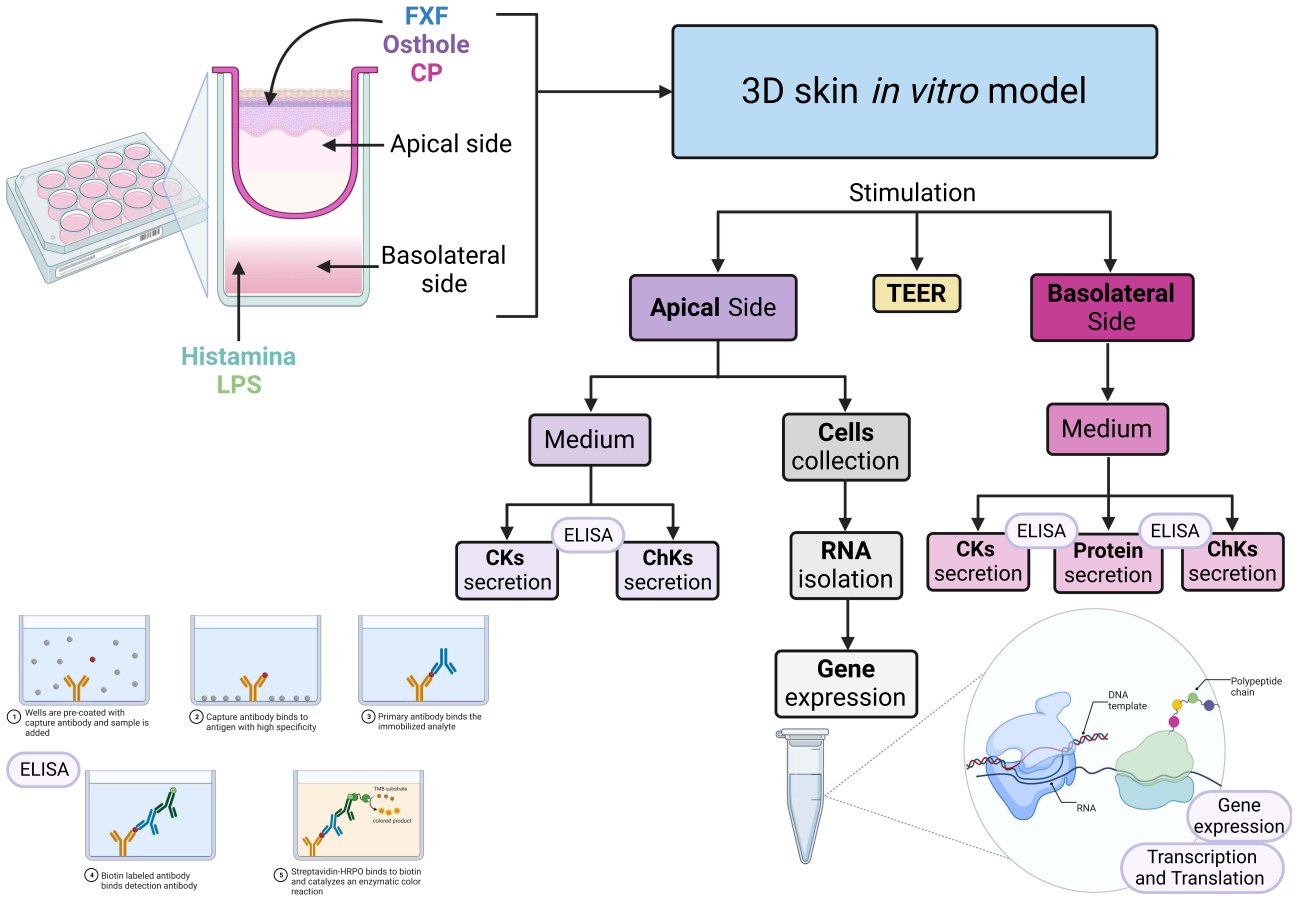

Figure 2 Experimental setup. Created with BioRender.com.

hours of incubation, different concentrations of CP (0.125 mg/mL, 0.50 mg/mL) and osthole (0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.50 mg/mL) were added to the apical side of the insert. CP and osthole were also added to the insert without prior stimulation with LPS. After 72 hours of incubation, the media (basolateral and apical sides) were removed for cytokine secretion analysis and 3D organotypic skin cells were removed from the insert for total RNA isolation and reverse transcription. Previous experience has shown that this is the time during which the 3D culture functions properly without structural changes, and during which changes in gene expression and interleukin secretion can be detected.

Analysis of Cytokine Levels

The concentrations of IL-4 and IL-13 were analyzed in the collected media after incubation of the 3D skin cells with different substances. The concentration of IL-4 and IL-13 in the media was tested using an enzyme-linked immunosorbent assay (ELISA) from R&D System (Minneapolis, USA; IL-4 - Cat. No. D4050, IL-13 - Cat. No. D1300B). The analysis was performed according to the manufacturer's protocol. The samples were tested in groups of four. The results were standardized by comparison with a standard curve.

RNA Isolation and Reverse Transcription

Total RNA was isolated according to the protocol described in Kordulewska et al 2022.[28] The concentration and purity of the extracted RNA (A260/A280) was determined using the NanoDrop ND −1000 spectrophotometer (NanoDrop Tech., Inc. Wilmington, DE, USA). Purified RNA was processed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA, cat. no. 4368814) according to the manufacturer's instructions in a Master Cycler Gradient Thermocycler (Eppendorf, Hamburg, Germany).

Quantitative Real-Time PCR (qPCR) and Data Analysis

The changes in the expression of IL-4Rα, H4R, TRPV1, TRPV4, and TRPM8 in NHEK and organotypic 3D skin were examined. YWHAZ was used as a reference gene to normalize the mismatch in mRNA quantity. The sequences of the individual primers used in the experiment are listed in Table S1. The qPCR was performed with the QuantStudio™ 3 Real-Time PCR System using the FastStart Essential DNA Green Master Kit (Roche Diagnostics, Basel, Switzerland, cat. no. 06402001). For each reaction, 5 ng of cDNA was given and qPCR was performed in triplicate as follows: Denaturation at 95°C for 10 min, amplification and quantification were repeated 45 times (95°C for 20s, 60°C for 20s, and 72°C for 20s with a single fluorescence measurement), melting curve at 60–95°C with a heating rate of 0.1°C per second and continuous fluorescence measurement, final cooling to 4°C. A negative control without cDNA and an inter-run calibrator were included in each assay. Gene expression was analyzed according to Pfaffl.[46] Results were scaled by the expression level of the control, which was set as one.

Statistical Analysis

Data analysis and visualization were performed using GraphPad Prism software version 9 (GraphPad Software, San Diego, CA, USA) and presented as mean ± standard deviation. Ordinary two-way tests ANOVA with Šídák multiple comparison tests were used to examine differences between quantitative values. The statistical significance level was set at a p value < 0.05.

Results

OST Inhibits the Th2 Cell Response (IL-4 and IL-13) in Histamine- or LPS-Treated NHEK Cell Lines and in the 3D Skin Model

Inflammatory mediators significantly decreased the secretion of IL-4 in the NHEK cell line (Figure 3A) and in the 3D skin model (Figure 3B). In the NHEK cell line stimulated with histamine, the highest concentration of IL-4 was observed after treatment with OST at a dose of 0.125 mg/mL. The secretion of IL-4 was lower and at a similar level in NHEK cells after treatment with hist/ CP and OST at a dose of 0.5 mg/mL. The same trend was observed in the keratinocyte cell line after the use of LPS as an inflammatory mediator. It was confirmed that the higher dose of OST effectively inhibited the secretion of IL-4 in the NHEK cell line. In a 3D skin model in which inflammation was induced by histamine, the stronger inhibition of IL-4 secretion was observed by using OST at a dose of 0.5 mg/mL, and the effect was like that observed by using CP. In 3D skin samples stimulated with LPS, OST was found to significantly decrease the secretion of IL-4, but no dose effect was observed.

OST significantly reduced the secretion of IL-13 in the NHEK cell line (Figure 3C) and the 3D skin model (Figure 3D) stimulated with histamine and LPS. It was found that the use of OST at a dose of 0.5 mg/mL reduced the secretion of IL-13 more effectively than a dose of 0.125 mg/mL, and the effect was comparable to CP at a dose of 0.5 mg/mL. The same IL-13 secretion profile after treatment with OST was demonstrated in the NHEK cell line and in the 3D skin model.

It should also be noted that higher secretion of IL-4 and IL-13 was observed after using LPS as an inflammatory mediator in the NHEK cell line than after treating the cells with histamine.

OST Regulated the Expression of IL-4Rα, H4R, TRPV1, TRPV4, and TRPM8 mRNA in the Histamine/LPS-Induced NHEK Cell Line and in the Organotypic 3D Skin Model

The doses of OST used in this study caused a change in histamine receptor 4 expression in the NHEK cell line and 3D skin model after stimulation with histamine and LPS (Figure 4A and B). In the histamine treated NHEK cell line, it was found that the use of both doses of OST significantly decreased histamine 4 receptor expression (Figure 4A). However, OST at the dose of 0.5 mg/mL was comparable to that used in this experiment CP. The level of gene expression for this receptor was like that of the control sample. In the LPS-stimulated NHEK cell line, OST at a dose of 0.5 mg/mL proved to be most effective in reducing histamine 4 receptor gene expression. It can be assumed that this dose of OST lowered the expression level of these genes to the level measured in the control sample. Moreover, this dose of OST was more

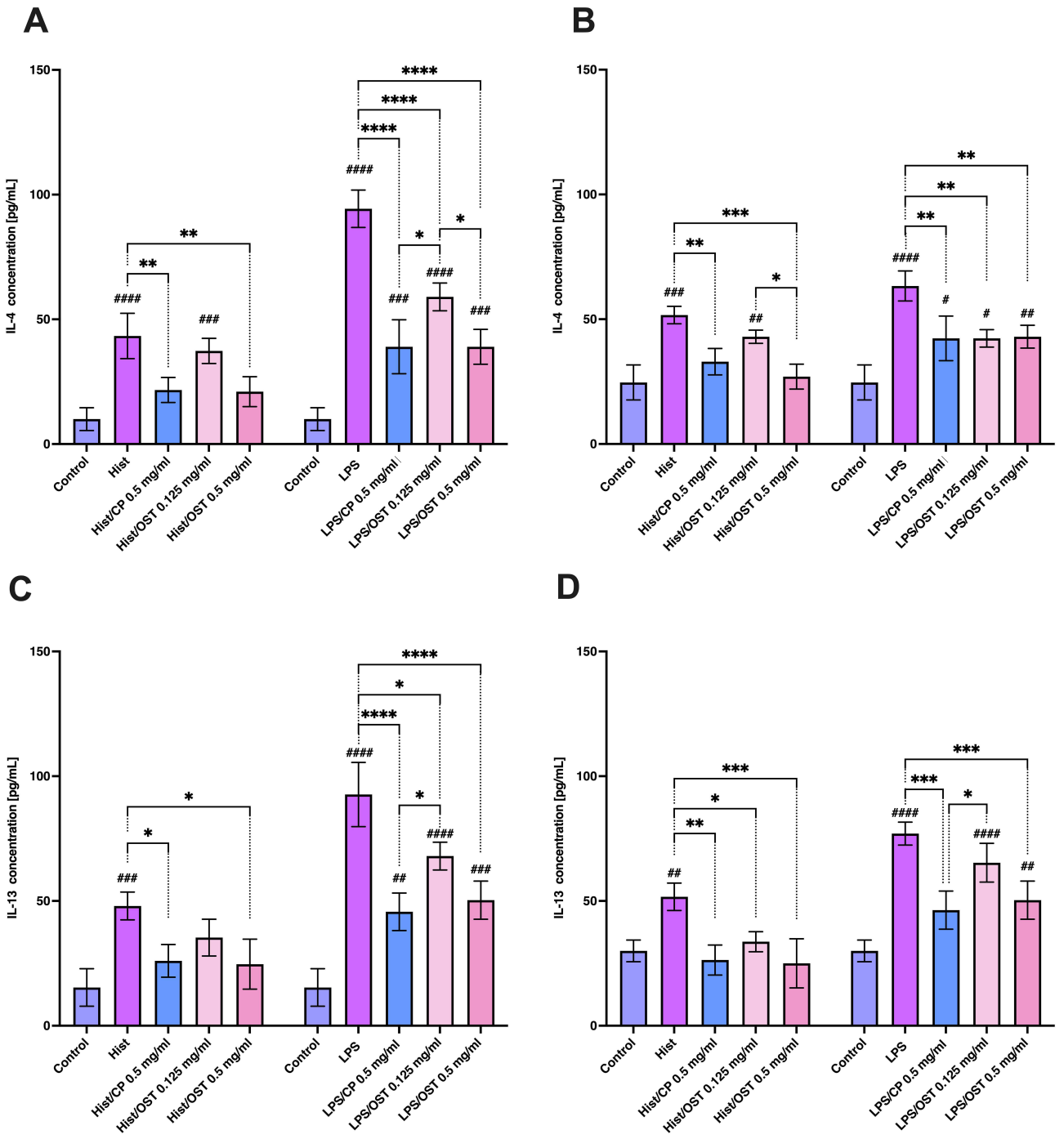

Figure 3 The level of IL-4 (**A** and **B**) and IL-13 (**C** and **D**) after incubation with histamine (Hist; 100 μg/mL), lipopolysaccharides (LPS; 2 μg/mL) alone and in mixtures with osthole (OST; 0.125 and 0.5 mg/mL) and clobetasol propionate (CP; 0.5 mg/mL) in Normal Human Epithelial Keratinocytes (NHEK; (**A** and **C**)) and prepared model of 3D skin (3D skin; (**B** and **D**)). The horizontal line shows the mean and the bars show the standard deviation. Statistically significant differences (Two-way ANOVA with Tukey's multiple comparisons test) compared to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) and to cells treated with histamine or LPS ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$) are marked.

Abbreviation: ns, non- significant.

effective in inhibiting the expression of histamine receptor genes than CP. When OST was used in the 3D skin model stimulated with histamine and LPS, the same effect was observed. The use of OST at the dose of 0.5 mg/mL inhibited the expression of histamine 4-receptor genes more than the dose of 0.125 mg/mL. The efficacy of the higher dose of OST was comparable to that of CP (Figure 4B).

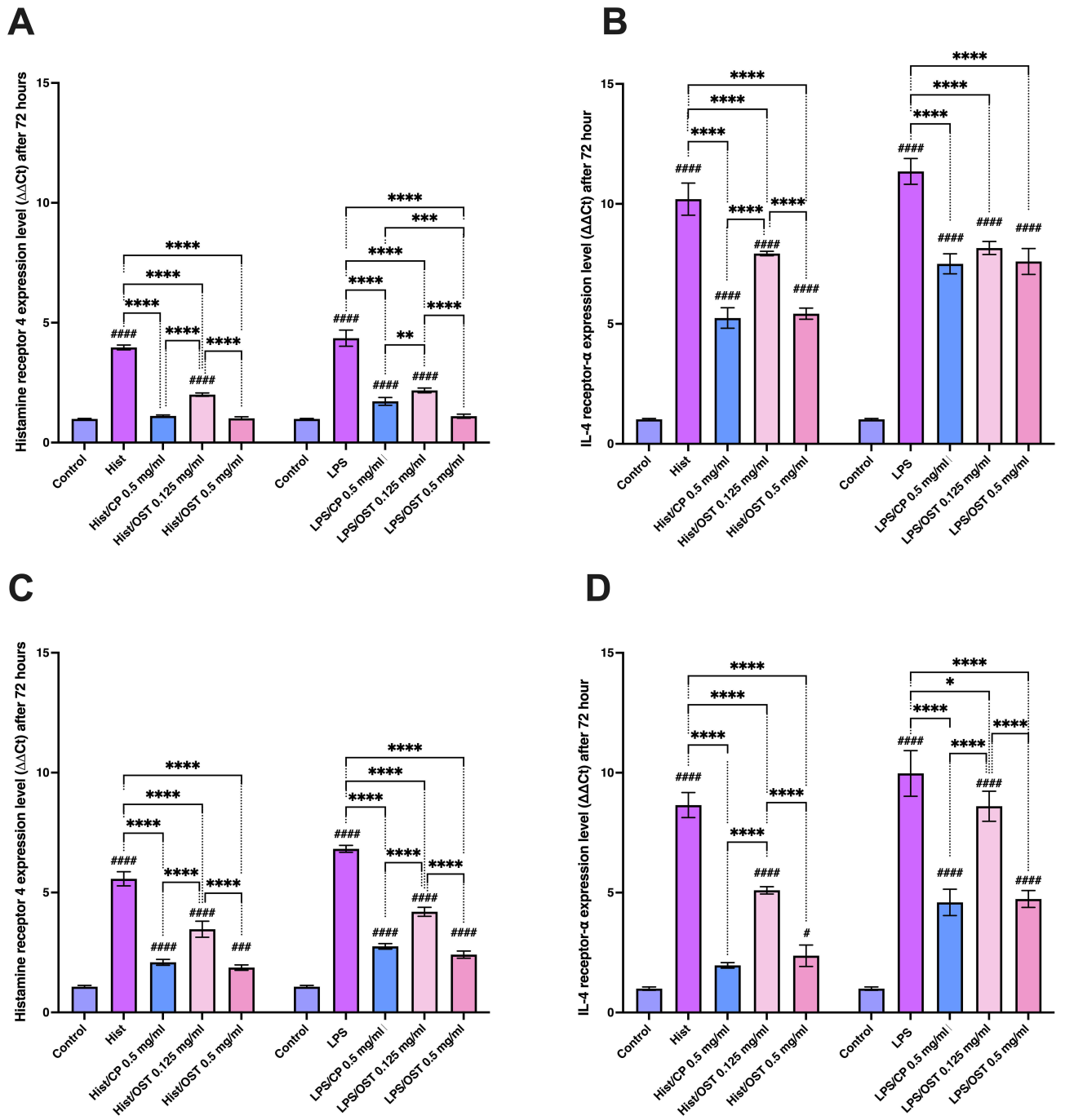

Figure 4 Histamine receptor 4 (**A** and **B**) and IL-4 receptor-α (**C** and **D**) gene expression level after incubation with histamine (Hist; 100 µg/mL), lipopolysaccharides (LPS; 2 µg/mL) alone and in mixtures with osthole (OST; 0.125 and 0.5 mg/mL) and clobetasol propionate (CP; 0.5 mg/mL) in Normal Human Epithelial Keratinocytes (NHEK; (**A** and **C**)) and prepared model of 3D skin (3D skin; (**B** and **D**)). The horizontal line shows the mean and the bars show the standard deviation. Statistically significant differences (Two-way ANOVA with Tukey's multiple comparisons test) compared to control (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) and to cells treated with histamine or LPS ($^{\#}$p < 0.05, $^{\#\#\#}$p < 0.001, $^{\#\#\#\#}$p < 0.0001) are marked.
Abbreviation: ns, non- significant.

In the next part of the experiment, the expression level of the IL-4α receptor was examined in the NHEK cell line and in a 3D skin model treated with histamine and LPS (Figure 4C and D). In the NHEK cell line, OST at a higher dose (0.5 mg/mL) inhibited IL-4α receptor gene expression more effectively than the lower dose (0.125 mg/mL). The higher dose of OST used had comparable efficacy to CP (Figure 4C). OST was found to decrease the expression of IL-4α receptor in the 3D skin model after stimulation by histamine and LPS (Figure 4D). In the samples of the 3D skin model

in which histamine was used as an inflammatory mediator, administration of a higher dose of OST had a significant effect on reducing the expression of the IL-4α receptor. Treatment of the 3D skin model stimulated with LPS with OST resulted in a decrease in the expression of the IL-4α receptor. In this case, no effect of OST dose was observed.

In this study, the effect of OST on the expression of three genes was investigated in the NHEK cell line and 3D skin model (Figure 5). It was found that in the NHEK cell line stimulated with histamine, both doses of OST used resulted in significantly decreased expression of the TRPV1 gene. OST at a dose of 0.5 mg/mL inhibited TRPV1 expression more efficiently than CP (Figure 5A). In the LPS-stimulated NHEK cell line, OST at a lower dose resulted in a similar reduction in TRPV1 expression as CP. Moreover, it was observed that a higher dose of OST was even more effective in reducing TRPV1 expression and was comparable to the expression level of this gene in control cells. A similar profile of downregulation of TRPV1 expression during OST treatment was observed in the 3D skin model (Figure 5B). In the 3D skin model stimulated with histamine, a higher dose of OST resulted in more effective inhibition of TRPV1 expression than CP. In contrast, in the 3D skin model stimulated with LPS, a lower dose of OST was found to inhibit TRPV1 expression at a similar level as CP, whereas application of a higher dose of OST resulted in a greater reduction in TRPV1 expression than that used by CP.

OST significantly decreased the expression of TRPV4 in the NHEK cell line (Figure 5C) and in the 3D skin model (Figure 5D) stimulated with histamine and LPS. In the NHEK cell line stimulated with both inflammatory mediators, it was found that OST at a dose of 0.125 mg/mL had a comparable effect in inhibiting TRPV4 expression as CP (Figure 5C). On the other hand, it was found that the expression of TRPV4 was lower after application of a higher dose of OST to the cells compared with treatment with CP. In the 3D skin model stimulated by histamine, it was observed that the application of OST at a dose of 0.5 mg/mL was much more effective in reducing the TRPV4 expression level than the lower dose of OST and CP. It should be noted that the expression level of this gene was comparable to that of the control sample when a higher dose of OST was applied (Figure 5D). In contrast, in the LPS-stimulated 3D skin model, the lower dose of OST was found to have a similar effect in reducing TRPV4 expression as CP. In contrast, application of a higher dose of OST resulted in a greater decrease in TRPV4 expression than CP.

OST Increases TRPM8 mRNA Expression in Histamine/LPS-Induced NHEK Cell Line and in the Organotypic 3D Skin Model

Application of OST to histamine- and LPS-stimulated NHEK cell lines and a 3D skin model resulted in a significant increase in the expression level of TRPM8 (Figure 5E and F). It was found that OST at low doses in the NHEK cell line resulted in a comparable increase in TRPM8 expression as CP (Figure 5E). Moreover, it was observed that a higher dose of OST more effectively increases the expression of TRPM8. In the 3D skin model treated with histamine and LPS, the same effect of OST on TRPM8 expression was observed (Figure 5F). It was found that a lower dose of OST increase TRPM8 expression at a similar level as CP, whereas the application of a higher dose of OST caused a greater increase in TRPM8 expression than the one used CP.

Discussion

AD is the name for a multifaceted, chronic and recurrent inflammatory skin disease that affects people on all continents and in all age groups. AD patients have chronic skin eczema and struggle with constant itching and sores. It is very uncomfortable for the patients and the healing therapies are not fully effective because the mechanisms underlying chronic itching are not understood[47]. For this reason, research should be constantly developed in the search for a new agent that alleviates the symptoms of AD.[47]

AD often manifests as mild to very persistent, itchy and open, blotchy lesions on the skin associated with increased skin sensitivity. The T helper 2 (Th2) response (mainly IL-4 and IL-13) is mainly associated with the acute and subacute phase, which was also confirmed in our studies where the factors contributing to the conditions of AD caused an increase in the secretion of the above ILs. TRP cation channels are a very important and under-researched aspect associated with dermatoses leading to skin pain and itching. The circuits in AD may play an important role. However, the stimulation of IL and the changes in gene expression in TRP channels in keratinocytes and organotypic 3D skin are underestimated.

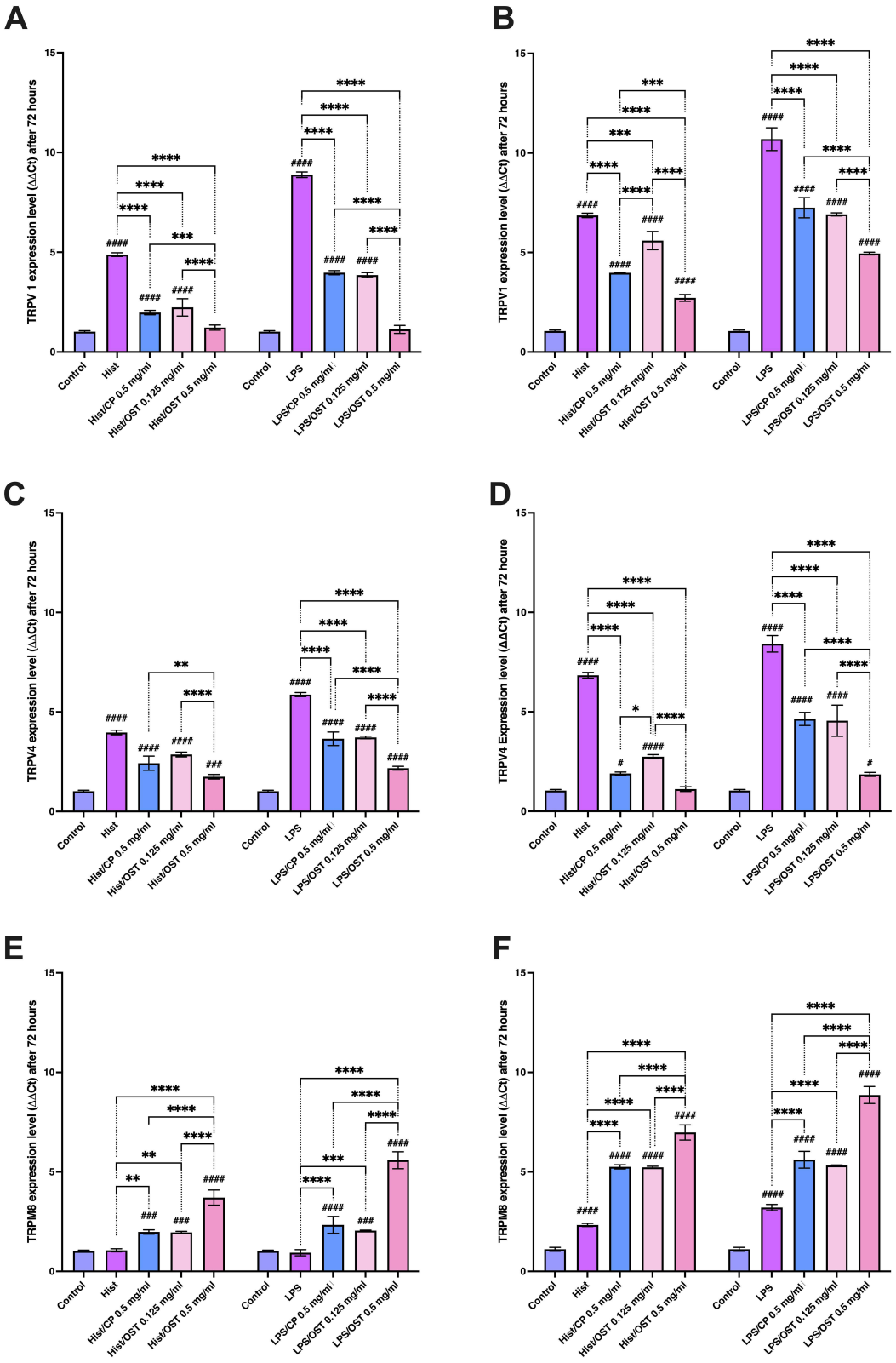

Figure 5 TRPV1 (**A** and **B**), TRPV4 (**C** and **D**) and TRPM8 (**E** and **F**) gene expression level after incubation with histamine (Hist; 100 μg/mL), lipopolysaccharides (LPS; 2 μg/mL) alone and in mixtures with osthole (OST; 0.125 and 0.5 mg/mL) and clobetasol propionate (CP; 0.5 mg/mL) in Normal Human Epithelial Keratinocytes (NHEK; (**A, C** and **E**)) and prepared model of 3D skin (3D skin; (**B, D** and **F**)). The horizontal line shows the mean and the bars show the standard deviation. Statistically significant differences (Two-way ANOVA with Tukey's multiple comparisons test) compared to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) and to cells treated with histamine or LPS ($^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.001$, $^{\#\#\#\#\#}p < 0.0001$) are marked.

Abbreviation: ns, non- significant.

Recent confirmations indicate that Th2-related ChKs modulate gene expression in TRP channels and thereby promote inflammation. The results obtained in the present manuscript suggest new conclusions:

(i) the increased secretion of IL-4 and IL-13 modulates the expression of the mRNA genes TRPV1, TRPV4 and TRPM8. The observed differences may be caused by other ChKs of the Th2 subfamily, eg, IL-25 and/or IL-33, which directly increase the secretion of IL-13, IL-31, and TSLP in the skin.
(ii) OST reduced the mRNA expression of TRP channels (TRPV1, TRPV4,) IL-4Rα, H4R and induced by inflammatory factors that reproduce the AD state (histamine, LPS). In addition, the effect of OST significantly reduced the activity of the tested TRP channels by the commonly used drug - glucocorticosteroid - CP.
(iii) OST stimulated mRNA expression of TRPM8 induced by inflammatory factors that reproduce the condition of AD (histamine, LPS). This could be an indication of the antipruritic effect of OST.
(iv) OST reduced the secretion of IL-4 and IL-13 after cell stimulation with inflammatory factors (histamine, LPS) that cause AD. Also in this variant, OST significantly reduced the secretion of the tested IL's compared to the control sample - A drug often associated with AD - glucocorticosteroid - CP.
(v) plants with medicinal properties should be the basis for production of new medicines[48,49] to which OST undoubtedly belongs as of now.

In summary, the results obtained clearly show that IL-4 and IL-13 can enhance the activity of TRP channels acting on mechanisms involved in inflammatory conditions, including pruritus, combining the effects with signaling cascades that we observe in AD. The results obtained confirm that the occurring disturbances in the modulation of TRP channels, which is dependent on Th2, can inhibit the transition from acute to chronic state in dermatoses. These conclusions can be used in the search and development of an effective new therapy and in the optimization of treatment conditions for patients. As the results show, OST could be an excellent candidate for further research into a future drug for AD. In addition to stimulating IL's, it also acts directly to reduce mRNA gene expression in TRP channels, which are thought to be critical for persistent skin inflammation.

IL-4 and IL-13 are currently considered potent mediators of type 2-associated immune inflammation, which is clearly manifested in Alzheimer's disease. IL-4 is known to exert very similar biological functions to IL-13, which can be explained by its ability to signal via the IL-4 type 2 receptor (R). This receptor consists of IL-4Rα, which is linked to IL-13Rα1. Unfortunately, the role of IL-4R type 2 is not yet clear.[50]

In our studies, the levels of IL-4 and IL-13 were significantly reduced in both the NHEK line and the 3D skin under the influence of OST. The factors modulating the state of AD were inhibited by the effect of OST. Similar results were obtained by Fu and Hong (2019), in which the mRNA expression levels of IL-4 and IL-13 were significantly increased in DNCB-induced AD (AD model, mice were sensitized with 2,4-dinitrochlorobenzene DNCB), while they decreased after acute treatment.[51] Similar data were also noted by Chiang et al, Yang et al, Wang et al, who demonstrated the antiallergic effects of OST in asthmatic mice and investigated the immunomodulatory effects in dendritic cells (DCs) and T cells. These studies showed that oral administration of OST to BALB/c mice induced with ovalbumin (OVA) reduced the production of Th2 ChKs (mainly IL −4, IL −5, and IL −33).[33,34,52] Similar results were published by Shen et al, where treatment under the influence of OST after OVA induction statistically significantly reduced the previously elevated serum IgE and ChK levels (IL −4, IL-5, IL-6, IL-13). The exception was IL-10 in bronchoalveolar lavage fluid (BALF).[53]

In our study, a similar sequence of results was observed with respect to of IL-4 receptor α expression. OST significantly decreased the expression of the studied receptor, suggesting that the decrease in expression of the receptor by OST is directly proportional to the secretion of the proteins (IL-4 and −13). To date, there is no evidence that allows us to discuss the results with other authors, as studies of this type have not been performed in 3D skin model after OST stimulation. We wanted to test the IL-4 receptor α subunit because *dupilumab*, an AD drug, is a monoclonal antibody that targets the IL-4 receptor-α subunit and blocks both IL-4 and IL-13 signaling.[54] In our studies, OST also reduced the expression of receptors and the secretion of IL-4 and IL-13, which we believe is further evidence for the possibility of using OST as a treatment for AD. In addition, recent studies have shown that specific IL-13 antagonists, which include

tralokinumab and lebrikizumab, have a similar effect to dupilumab.[55–57] These results may suggest that IL-13 can be used as critical ChK's in dermatoses of varying severity.[58]

Based on the study by the team of Yang et al 2021, in which the researchers investigated the pharmacological effects of OST on TRPV1 and found suppression of histamine VUF8430 (histamine H4 receptor agonist)-induced scratching in mice,[59] we hypothesized, that the inhibition of histamine-dependent itch by OST could be caused by the activation and subsequent desensitization of TRPV1 and H4 receptor expression in the NHEK line, and 3D model skin, as histamine activates H1R and H4R in DRG neurons via the PLCβ/PKC pathway and/or the PLA2/lipoxygenase (LO) pathway and ultimately couples to TRPV.[60–62] The H4 receptor has been shown to be involved in the development of AD as it has properties that modulate the function of many immune cells in the skin (ie, T cells[63] and DCs).[64] As with the previous results, OST statistically significantly decreased the expression of H4R expression in both variants tested. Studies using 3D skin and OST in conjunction with H4R were not reported, but expression on keratinocytes cell line was confirmed as by other authors.[65] A similar relationship to this manuscript was found in the studies of Kordulewska et al, where OST decreased H4R expression in the Caco-2 line at each concentration tested.[35] Surprisingly, we found that H4R expression was higher under the influence of LPS than under that of histamine. The answer to these differences could be the fact that the skin of people with AD faces constant fluctuations and higher concentrations of histamine, which can desensitize the receptor to histamine even in vitro. This can also lead to patients responding less well to antihistamines and antiallergic medications. In addition, this difference may be preceded by proliferation of keratinocytes. The results obtained are also confirmed by studies by Glatzer et al, in which the researchers found in in vitro studies on human keratinocytes that by stimulating H4R they promoted the induction of proliferation of keratinocytes derived from people diagnosed with AD, but not from healthy people.[65] The significant difference between the study groups could be due to a different cellular response to histamine. AD -The keratinocytes of the patients may have higher expression of H4R than those of the control group. The results describing the difference in H4R expression in the Chinese population of people with AD suggest that regulation at the genetic level plays an important role, especially the polymorphism in the HRH4 gene.[66] However, the reasons for this are still unknown. There is only evidence of higher expression of the H4R gene in keratinocytes with dermatoses, which should be the subject of further investigation.

Itching, common in AD, is an annoying and agonising sensation caused by the activation of a subset of sensory neurons in the skin. TRP ion channels, including TRPV1, TPRV4, and TRPM8, which are expressed in sensory neurons or keratinocytes appear to be involved in itching associated with various pathological conditions.[67] Therefore, based on the literature data, we aimed to investigate the effect of OST on sensation and inhibition of TRP channels expression. While histamine released by MC's controls the histamine receptor TRPV1 directly or indirectly influences skin itch through many factors that cause skin pruritus.[61] As previously documented, TRPV1 directly mediates acute and chronic dermatoses induced by histamine or LPS.[68,69] Therefore, recent research has focused on the search for a selective TRPV1 inhibitor as a potential anti-inflammatory agent.[70] Our results confirm that OST inhibits TRPV1 gene expression. It is very important to note that TRPV is expressed in keratinocytes and not in sensory neurons.[67] It should be added at this point that Olmsted syndrome is a severe skin disease caused by the TRPV4 mutation. This mutation leads to increased activation and patients suffer from very severe and chronic itching.[71] It is therefore assumed that inhibition of TRPV4 could also be a promising pharmacological approach. In our studies, OST significantly reduced the mRNA expression of TRPV1 and TRPV4, and increased TRPM8 channels induced by histamine and LPS. This may indicate that it is an effective inhibitor of TRP channels and a suitable agent in anti-atopic drugs. Increased expression of TRPV1 in the skin of mice is observed in models of AD.[72,73] Scientific studies confirm the inhibition of TRPV1 activity by the molecule PAC −14028 in a mouse model of AD, caused by Dermatophagoides farina (Df) and oxazolone (OXZ). The symptoms typical of the dermatosis improved. Serum IgE levels, MC degranulation, clinical exacerbation of dermatitis, and persistent scratching.[74] AMD9810, one of the other TRPV1 antagonists, directly blocked the firing of sensory neuron. It also significantly reduced scratching in a model of acute inflammatory pruritus in mice induced by subcutaneous injection of Immepip into the neck of the animals.

Very interesting data were published in studies with HC030031, which is also classified as a TRPV1 antagonist. These results suggest that TRPV1 may be involved in itch signaling mediated by the H4[75] histamine receptor. This was also confirmed in our study, in which OST also significantly reduced its expression. In skin dermatoses, H4 receptor

antagonists showed not only antipruritic but also anti-inflammatory effects, which have been observed in many mouse models and human clinical studies. The conclusions drawn clearly indicate that the H4 receptor plays an important role in AD. At this point, it should be noted that the research results published by Oh et al are very similar to our research.

The studies showed that the increased expression of IL-13 in AD transgenic mice after administration of HC030031, which is classified as a TRPV1 agonist, significantly inhibited the itching caused by IL-13 and inhibited the expression of TRPV1 in the skin. However, itch in the skin was not completely eliminated, which may indicate that a mechanism independent of TRPV1 controls the pathogenesis of itch, which in turn is mediated by IL-31.[76] Stabilized TRPV signaling is necessary for the maintenance of epidermal barrier homeostasis. Stimulated activation of TRPV may be an example of a critical signaling cascade that occurs in the cells, and it may influence proper cell proliferation, thanks to which skin barrier formation, normal hair growth, release of immune mediators, etc. are possible. Changes in these activities in TRPV are observed in patients with atopic dermatitis and similar skin diseases. Mutations that impair the normal function of TRPV may alter the normality of hair growth in the dermis and lead to disruption of skin barrier homeostasis.[77,78] The inability to grow hair due to upregulation of TRPV may further exacerbate pruritus in AD patients.[79] It is hypothesised that upregulation of the TRPV channel in AD keratinocyte cells leads to increased pro-inflammatory levels in response to Th2 inflammation, which may be the reason for NF-κB activation.[80,81] Increased expression of NF-κB mRNA was confirmed in our previous studies, and OST significantly reduced it in 3D skin and skin cell lines.[28,29] It is very possible that the decreased expression of the TRPV4 channel after OST stimulation is related to the decreased activation of the NF-κB signalling and the reduction of inflammatory IL's. Furthermore, we believe that in human keratinocytes, PLCβ activation by the Gq/11 protein-coupled receptor tremendously enhances TRPV4 currents, and stimulation of the M1 acetylcholine receptor increases the sensitivity of TRPV channel activation.[82,83] Such a mechanism is also crucial for the activation of T cells at sites of inflammation, which causes sensitization of the TRPV channel.

In our study, the expression of TRPM8 was significantly increased after incubation of the cells with OST. Thus, we confirmed the studies of other authors on its detection.[84] TRPM8 plays a role in non-noxious cold sensation. It is also activated by chemical compounds known to elicit cold sensations, such as menthol and icilin.[85] However, unlike other TRP channels, TRPM8 activity suppresses rather than elicits itch in most cases. Local cooling has been used to reduce itch because cooling reduces nerve excitability and conduction velocity, slowing some itch transmission pathways, such as the one involving TRPV1. However, cold temperatures and menthol also stimulate sensory neurons involved in TRPM8.[86] Palkar et al showed that cold successfully inhibits both histaminergic and non-histaminergic itch transmission pathways and that this mechanism requires the activation of TRPM8 channels or TRPM8-expressing afferent neurons. Although not pruriceptors, TRPM8 neurons are thought to play a role in itch suppression by participating in a spinal interneuron circuit involving B5-I neurons. These inhibitory spinal interneurons receive input from menthol-sensitive afferents and produce dynorphin, a neuropeptide known to suppress itch.[87] Menthol has been shown not to inhibit itch in mice lacking B5-I neurons, suggesting that these neurons play a role in the antipruritic effects associated with activation of TRPM8.[88] Cooling has been shown to inhibit chronic itch, making TRPM8 a potential target for antipruritic therapy. In our studies, factors that induce the AD condition did not lead to significant changes in TRPM8 expression, whereas OST significantly increased expression. This could mean that it has properties that influence its activation, which manifests itself in the desired biological effect, ie, suppression of the inflammatory response in atopic skin. Similar results were obtained by the team of Kim et al. The TRPM8 receptor was expressed primarily in bronchial epithelial cells at both mRNA and protein levels with statistical significance. Activation of TRPM8 receptors by menthol was associated with increased expression of inflammatory cytokines IL-4, 6, 8, 13, 33 and treatment with TCM attenuated the expression of inflammatory cytokines.[89]

In summary, OST has various biological activities:[90] anti-inflammatory[27,91,92] antibacterial,[93] antiviral,[36] antifungal,[94,95] antioxidant,[96,97] antithrombotic,[98] antidiabetic,[99] antispasmodic,[100,101] and anti-tumor.[25,102,103] OST is used as an additive in food and cosmetics due to its antifungal and antioxidant properties.[104] Coumarins are considered as drug candidates with diverse pharmacological activities, benefiting from their attractive properties, such as low molecular weight, simple structure, high solubility in most organic solvents, low side effects, low drug resistance, high bioavailability, broad spectrum of activity, and better curative effect In addition, the results obtained, which are the first so far related to mRNA expression

of TRP channels in a 3D organotypic skin model, should be considered and used in the further phases of clinical studies on the efficacy of using OST as an active ingredient in drugs against atopic skin diseases. Of course, our study still has some limitations. 1) Given the direct link between TRP channels, interleukin secretion and OST stimulations, a genetically modified mouse model might provide more evidence. 2) We have theoretically confirmed the effect of TRP channel induction/inhibition, interleukin secretion and OST stimulations on the organotypic keratinocyte/3D model in vitro, but in vivo experiments are still lacking. 3) Although our work resulted in a significant decrease in gene expression of IL-4α, H4R, TRPV1, TRPV4 and an increase in TRPM8 in both the NHEK cell line and the organotypic 3D skin model. How Osthole inhibits the entire pathway is still unclear and further research in this area is needed.

Furthermore, although the 3D skin system is considered a model and is well established in atopic dermatitis research, it is not a comprehensive organism that significantly interacts with each other. It is also important to add that atopic dermatitis may differ from person to person in terms of disease progression, effects of medications/agents and environmental factors, which has not been considered in the present manuscript.

Conclusion

Overall, these results show that Th2 cytokines enhance TRP channels under different inflammatory and pruritic conditions by different mechanisms and link this effect to different signaling cascades in AD. These findings reinforce the notion that disruption of Th2-driven modulation of TRP channels will inhibit the transition from acute to chronic AD and thus contribute to the development of effective therapeutics and optimization of treatment.

The results of this study are consistent with this concept and provide the first in vitro evidence of itch relief by an OST-containing emollient in atopic skin.

Abbreviations

AD, atopic dermatitis; ASD, autism spectrum disorders; BALB/c, mice induced with ovalbumin; BALF, bronchoalveolar lavage fluid; ChKs, chemokines; CP, clobetasol propionate; DCs, dendritic cells; Df, Dermatophagoides farina; ELISA, enzyme-linked immunosorbent assay; GPCRs, G protein-coupled receptors; H1-H4, histamine receptors 1-4; IL's, interlukins; LO, lipoxygenase; LPS, lipopolysaccharide; MC's, mast cells; NHDF, Normal Human Dermal Fibroblasts; NHEK, Normal Human Epidermal Keratinocytes; OST, osthole; OVA, ovalbumin; OXZ, oxazolone; PBMCs, peripheral blood mononuclear cells; TCS, topical corticosteroid; TH2, lymphocytes Th2 helper; TRP, Transient receptor potential channels; TRPM8, Transient Receptor Potential Melastatin Subfamily 8; TRPV1, Transient Receptor Potential Vanilloid type 1; TRPV4, Transient Receptor Potential Vanilloid 4; VUF8430, histamine H4 receptor agonist; YWHAZ, Tyrosine 3-Monooxygenase.

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Disclosure

The authors report no conflicts of interest in this work.

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