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# First evidence of dermo-protective activity of marine sulfur-containing histidine compounds

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ABSTRACT

Among natural products, ovothiol (ovo), produced by marine invertebrates, bacteria, and microalgae, is receiving increasing interest for its unique antioxidant properties. Recently, ovo has been shown to exhibit anti-inflammatory activity in an *in vitro* model of endothelial dysfunction and in an *in vivo* model of liver fibrosis.

The aim of this study was to evaluate the effect of ovo and its precursor 5-thiohistidine (5-thio) in comparison with ergothioneine (erg), in human skin cells and tissues upon inflammation. We used both an *in vitro* and *ex vivo* model of human skin, represented by a keratinocytes cell line (HaCaT) and skin biopsies, respectively. We observed that ovo, 5-thio, and erg were not cytotoxic in HaCaT cells, but instead exerted a protective function against TNF-α -induced inflammation. In order to get insights on their mechanism of action, we performed western blot analysis of ERK and JNK, as well as sub-cellular localization of Nrf2, a key mediator of the anti-inflammatory response. The results indicated that the pre-treatment with ovo, 5-thio, and erg differently affected the phosphorylation of ERK and JNK. However, all the three molecules promoted the accumulation of Nrf2 in the nucleus of HaCaT cells. In addition, gene expression analysis by RTqPCR and *ELISA* assays performed in *ex vivo* human skin tissues pre-treated with thiohistidines and then inflamed with IL-1β revealed a significant downregulation of *IL-8*, *TNF-α* and *COX-2* genes and a concomitant significant decrease in the cytokines IL-6, IL-8 and TNF-α production. Moreover, the protective action of ovo and 5-thio resulted to be stronger when compared with dexamethasone, a corticosteroid drug currently used to treat skin inflammatory conditions.

Our findings suggest that ovo and 5-thio can ameliorate skin damage and may be used to develop natural skin care products to prevent the inflammatory status induced by environmental stressors and aging.

## 1. Introduction

Skin represents the largest organ in the human body and the outermost barrier against external insults like pathogenic microorganisms [1,2] and ultraviolet (UV) rays [3]. Several skin diseases like psoriasis, dermatitis, infections, as well as lesions caused by the excessive exposure to UV radiation, are characterised by a widespread inflammatory state of the epidermal layer, and especially of keratinocytes [1]. In particular, the skin tissue is populated by numerous sentinels of the immune system, i.e. macrophages, lymphocytes T $CD4^+$ and dendritic cells, which, once activated, release pro-inflammatory cytokines, like interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor (TNF-α), known to play a key role in mediating cellular damage and the pathogenesis of chronic inflammatory diseases [4–7]. At the molecular level, TNF-α and interleukin 1β (IL-1β) mediate the activation of the transcription factor NF-κB, which in turn induces the transcription of several pro-inflammatory genes, also through the activation of mitogen-activated protein kinases (MAPKs) [8–10], including the

*Abbreviations:* 5-thio, 5-thiohistidine; erg, ergothioneine; GSH, glutathione; IL-1β, interleukin 1β; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; Nrf2, NF-E2-related factor-2; ovo, ovothiol.

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extracellular signal-regulated kinase (ERK), c-JNK N-terminal kinase (JNK) and p38/MAPK [11–15]. In this context, the hyper production of reactive oxygen species (ROS) is considered one of the key inducers of skin damage [13,16,17]. Indeed, ROS are known to induce ERK1/2 activation, its consequent translocation into the nucleus, and the activation of transcription factors, like the nuclear factor erythroid 2-related factor-2 (Nrf2). On its turn, Nrf2 is responsible for modulating the expression of redox sensitive genes [18–24], and inhibiting the transcription of pro-inflammatory mediators including cyclooxygenase 2 (COX-2) [25].

In the last decades, skin health has also been more threatened due to the rising of anthropogenic pressure, i.e. the increased exposures to UV radiation and chemical pollutants [26]. These environmental stressors contribute to accelerate skin aging and carcinogenesis, mainly through ROS production [27]. All these concerns, together with the growing global attention for environmental sustainability, have prompted the research of natural and eco-friendly bioactive compounds, to improve human skin health without impact on the environment [28]. In this context, oceans offer a huge reservoir of bioactive molecules, whose molecular structures and biological properties have inspired the synthesis and use of different pharmaceutical products [29]. Moreover, many marine compounds have been shown to offer great potential for the development of skin care products, when used in sunscreens and antiaging formulations [30].

Among marine derived natural products endowed with promising antioxidant and anti-inflammatory properties are sulfur-containing histidine derivatives, first isolated from marine invertebrates as free methylated forms or as part of larger molecules [31–35], and more recently found also in some proteobacteria and microalgae [36–39]. In particular, π-methyl-5-thiohistidine, commonly named ovothiol (ovo), due to its first discovery in sea urchin eggs [40] has been shown to exhibit anti-inflammatory properties in an *in vitro* model of human endothelial dysfunction [41] and in an *in vivo* model of liver fibrosis [42,43]. Moreover, ovo and its desmethylated precursor 5-thiohistidine (5-thio), have been discovered to inhibit the activity of γ-glutamyl transpeptidase, an enzyme involved in glutathione (GSH) metabolism and maintenance of redox homeostasis in human cancer cells [44,45]. Interestingly, another sulfur containing histidine, N-α-trimethyl 2-thiohistidine, named ergothioneine (erg), mainly produced in fungi and cyanobacteria [46], has been reported to protect human keratinocytes from UVA-induced damage [47].

The aim of this study was to evaluate the potential of ovo and 5-thio to protect the skin against inflammatory processes. We used both an *in vitro* model of human keratinocytes (HaCaT) and an *ex-vivo* model of human skin tissue, in order to test the cytotoxicity of ovo and 5-thio and their potential anti-inflammatory properties in comparison with erg, and dexamethasone, a corticosteroid drug currently used to treat skin disorders.

In order to identify the mechanism of action of such molecules, we assessed the protein expression of ERK, JNK and Nrf2 in the cytosol, as well as we evaluated Nrf2 translocation into the nucleus in HaCaT cells. In addition, we evaluated the inflammation-induced expression of the genes encoding the cytokines IL-6, IL-8, TNF-α, and COX-2 in human skin tissues, as well as their release into the medium. Overall, our experiments shed light on the efficacy of 5-thiohistidine derivatives as novel protective agents against skin inflammatory conditions.

## 2. Materials and methods

### 2.1. Cell cultures and compounds availability

The immortalized human skin keratinocyte cell line (HaCaT cells) was kindly provided by Prof. Tiziana Angrisano (Department of Biology, University of Naples Federico II). HaCaT cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$ in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% of heat-inactivated Fetal Bovine Serum (FBS; Gibco), 1% glutamine and 1% antibiotics (100 U/ml Penicillin and 100 μg/ml Streptomycin; Gibco). Ovothiol (ovo) was purified from sea urchin eggs, as previously described [40]; 5-thiohistidine (5-thio) was kindly provided by Prof. Florian Seebeck from University of Basel; ergothioneine (erg) was purchased by Sigma Aldrich.

### 2.2. Cell viability assays

To test the cytotoxicity of the molecules, HaCaT cells were plated in 384-multiwell plates ($2 \times 10^3$ cell/well) by a pipetting robot (Freedom Evo-2200 Liquids Handler, Tecan, Männedorf, Switzerland) and treated with ovo, 5-thio, and erg, at the concentrations ranging from 2 to 100 μM for 24, 48 and 72 h. Cell viability was assessed by resazurin-based assays (CellTiter-Blue® Cell Viability Assay, Promega, Madison, WI, USA) according to the manufacturer's recommendations using a Spark® multimode microplate reader (TECAN). The fluorescence was measured at $560_{Ex}/590_{Em}$ nm. All experiments were performed in triplicate. The effect of molecules on cell viability was assessed as the percentage of viable cells compared to not-treated control cells, which were arbitrarily assigned viability of 100%. To test the effect of the molecules upon inflammation, HaCaT cells were plated in 96-well plates ($5 \times 10^3$ cell/well), pre-treated with ovo, 5-thio and erg at concentrations ranging from 2 to 10 μM for 24 h, and incubated with TNF-α at four different concentrations (1, 2, 5 and 10 ng/ml) for 24 h to induce inflammation. Cell viability was assessed by CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) (Promega, Madison, WI, USA). The absorbance was measured at 540 nm using the microplate reader. The effect of TNF-α on cell viability was assessed as the percentage of viable cells compared to not-treated control cells, which were arbitrarily assigned viability of 100%. All experiments were performed in triplicate.

### 2.3. Preparation of total, cytosolic and nuclear protein extracts from HaCaT cells

HaCaT cells were seeded in 6-well plates ($5 \times 10^5$ cells/well) and pre-treated with ovo, 5-thio and erg at a concentration of 2 μM for 24 h and subsequently exposed to 10 ng/ml TNF-α for other 24 h, and compared to not treated cells (only medium) or treated only with TNF-α. To obtain total protein extracts, cells were washed with cold PBS and resuspended in RIPA lysis buffer (1X) containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 10% SDS, phosphatase, and protease inhibitor cocktail (Roche). Cell homogenates were centrifuged at 13000 g for 5 min at 4 °C, and the supernatant was used as total protein extract. To obtain the cytosolic and nuclear extracts, cells were resuspended in lysis buffer containing 10 mM Hepes (pH8.0), 0.1 mM EDTA, 10 mM KCl, 100 mM EGTA, 1 mM DTT, 2.0 mg/ml leupeptin, 2.0 mg/ml aprotinin, NP-40 10%, phosphatase and protease inhibitor cocktail (Roche). The cells were allowed to swell on ice for 15 min. The samples were vortexed every 5 min for 20 min. The homogenates were centrifuged for 5 min at 12.000 g and the supernatant was used as the cytosolic extract. The nuclear pellet was resuspended in cold extraction buffer containing 20 mM Hepes (pH 8.0), 1 mM EDTA, 400 mM NaCl, 1 mM EGTA, 1 mM DTT, 2.0 mg/ml leupeptin, 20 mg/mL aprotinin, phosphatase and protease inhibitor cocktail (Roche). The samples were centrifuged at 15000 g for 30 min and the supernatant was used as the nuclear extract. The protein content of the total, cytoplasmic, and nuclear extracts, was determined with the Bio-Rad protein assay reagent using bovine serum albumin as standard.

### 2.4. Western blot analysis

Total protein extracts were analyzed on 12% SDS/polyacrylamide using Laemmli buffer. Following electrophoresis proteins were transferred onto a PVDF (Millipore) membrane (Bio-Rad Trans-Blot Apparatus), and probed with the rabbit polyclonal antibodies: anti-ERK, anti-

phospho-ERK, anti-JNK, and anti-phospo-JNK (all from Cell Signalling), diluted 1:1000 (or 1:500 for anti-p-JNK) in non-fat dried milk 5% in PBS, and as an internal control the mouse anti-β-Actin monoclonal antibody (Santa Cruz Biotechnology, USA) diluted 1:1000 in PBS milk 5%. Cytoplasmic and nuclear extracts were analyzed using a mouse anti-Nrf2 monoclonal primary antibody (Santa Cruz Biotechnology) diluted 1:100 in milk 5%, and as an internal controls mouse anti-Histone H3 monoclonal primary antibody (Cell Signalling) diluted 1:500 in PBS milk 5%. All primary antibodies were incubated at 4 °C overnight. The appropriate secondary anti-mouse and anti-rabbit HRP-conjugated antibodies (Amersham) both diluted 1:10000 in milk 5%, were added at 37 °C for 1 h and immune-reactive proteins were detected using the ECL (WesternBrightTM detection kit ECL, Advansta, USA) according to the manufacturer's instructions. Immunopositive bands were analysed by densitometry using the Image J software. The effect of TNF-α on protein expression was assessed as fold induction compared to not-treated control cells, which were arbitrarily assigned protein expression value equal to 1. All experiments were performed in triplicate.

### 2.5. Nrf2 immunofluorescence

HaCaT cells were plated in 96-well plates ($5 \times 10^4$ cells/well). After pre-treatments with ovo, 5-thio, and erg, at a concentration of 2 μM and TNF-α 10 ng/ml, cells were washed with 1X PBS (Phosphate Buffer Saline, Gibco) and then fixed with 4% paraformaldehyde for 10 min. After three washes in 1X PBS, cells were permeabilized with 0.5% Triton X-100 for 10 min and washed with 1X PBS. Cells were then blocked with 10% FBS (Gibco) in 1X PBS for 30 min and then incubated overnight at 4 °C with anti-Nrf2 mouse monoclonal primary antibody (Santa Cruz Biotechnology, sc-365949) diluted 1:250 in 1.5% FBS. Cells were then washed three times with 1X PBS and then incubated with goat anti-mouse (Abcam, Alexa Fluor 488, ab150113) secondary antibodies for an additional 1 h, diluted 1:500 in 1.5% FBS. Then, cells were stained with 1 μg/ml DAPI (Life Technologies) for 10 min. Cells were examined with the DMI600 (Leica) fluorescence microscope and photographed using a DFC360-FX camera (Leica). Each experiment was performed in triplicate.

### 2.6. Ex vivo human skin assays

Human skin tissues were obtained from patients undergoing cosmetic surgery at the FACCIA, Aesthetic Clinic, Lisbon, Portugal. This study was approved by the clinic Ethics Committee. All principles of the Declaration of Helsinki were followed and written informed consent was obtained from all patients. Skin tissue fragments of approximately 2 mm depth were collected into DMEM media and transported to the laboratory within the next 24 h. Upon arrival, 4 mm skin explants were obtained using a 4 mm biopsy punch (Integra-Miltex) and set to equilibrate for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$ in complete media: DMEM supplemented with 10% of heat-inactivated Fetal Bovine Serum (FBS; Gibco), 1% glutamine and 1% antibiotics (100 U/ml Penicillin and 100 μg/ml Streptomycin; Gibco). Afterwards, skin explants (5 per well) were plated in a 24 well plate and cultured as described above in 0.5 ml of complete media supplemented with ovo, 5-thio, erg, or dexamethasone (DMX) at different concentrations for 24 h, and then treated with 10 ng/ml interleukin β1 (IL-1β) (Cat#200-01B, PeproTech) for further 24 h, to induce inflammation. As controls, explants were cultured without treatment. At the end of each experiment, skin explants were collected, washed twice with 1X PBS, immediately snap frozen in liquid nitrogen for RNA extraction, and the cell culture media collected for ELISA assays.

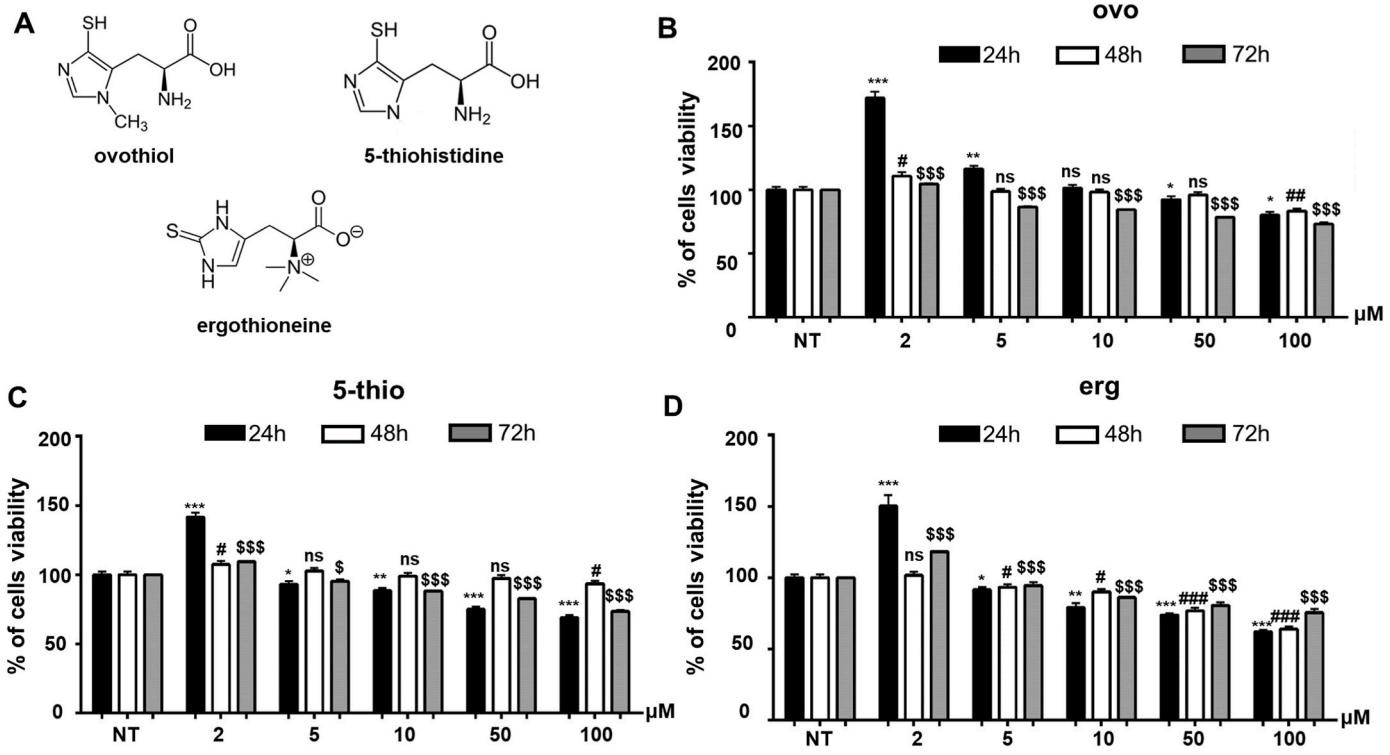


**Fig. 1. Effect of sulfur-containing histidine compounds on viability of HaCaT cells. (A)** Chemical structure of ovothiol, 5-thiohistidine, and ergothioneine. Cells were treated for 24, 48 and 72 h with ovo **(B)**, 5-thio **(C)** and erg **(D)** at five different concentrations (2, 5, 10, 50 and 100 μM) and cell viability was measured by a resazurin-based assay. Data are expressed as mean ± SD (standard deviation), $n = 3$. The significance was determined by the *t*-test student: * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) represent significance compared to not treated (NT) cells at 24 h; # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$) represent significance compared to NT cells at 48 h; $ ($p < 0.05$), $$ ($p < 0.01$) and $$$ ($p < 0.001$) represent significance compared to NT 72 h.

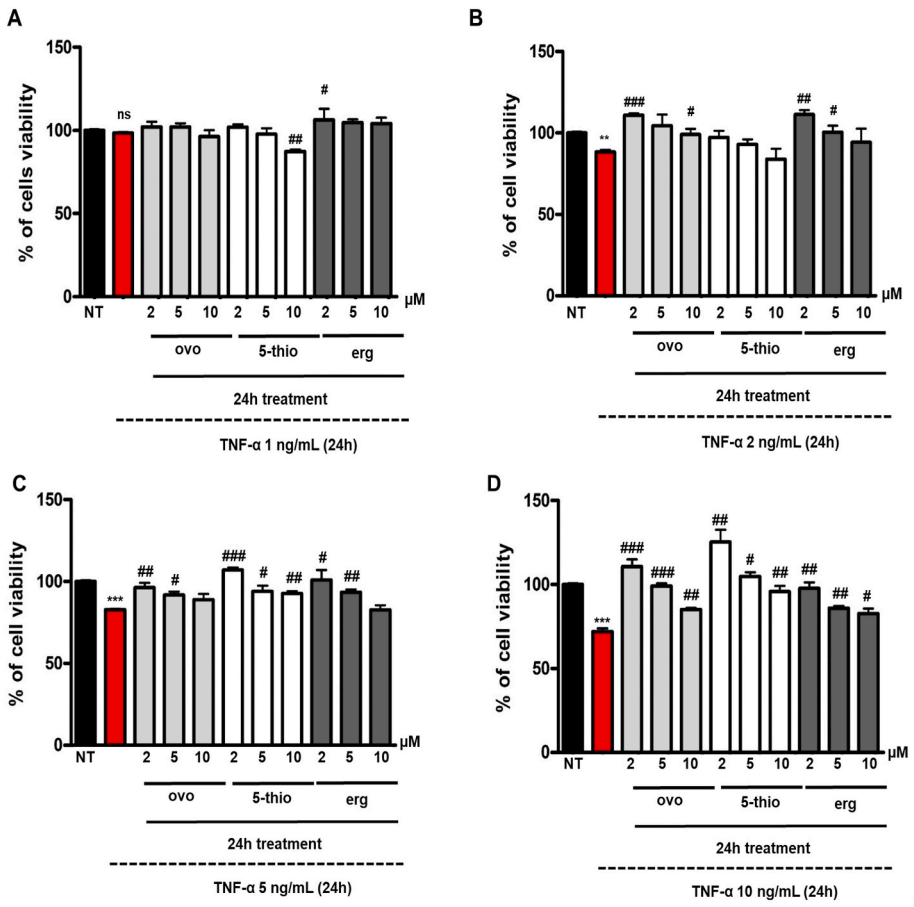


**Fig. 2. Effect of ovo, 5-thio and erg on TNF-α-induced inflammation in HaCaT cells.** Cells were pre-treated for 24 h with ovo, 5-thio and erg at three different concentrations (2, 5 and 10 μM) and inflammation was stimulated for 24 h with TNF-α at 1 **(A)**, 2 **(B)**, 5 **(C)** and 10 **(D)** ng/ml. Cell viability was measured by MTT assay. Data are expressed as mean ± SD, $n = 3$. The significance was determined by the *t*-test student: * ($p < 0.05$), ** ($p < 0.01$) *** ($p < 0.001$) represent significance compared to NT; $^{\#}$ ($p < 0.05$), $^{\#\#}$ ($p < 0.01$) $^{\#\#\#}$ ($p < 0.001$) represent significance compared to TNF-α treated cells. In all graphs, the red bar indicates the cells treated with TNF-α.

### 2.7. ELISA assay

Cell culture media were used for the quantification of IL-6, IL-8 and TNF-α using specific ELISA tests (Human IL-6, IL-8 and TNF-α Standard ABTS ELISA Development Kit; PEPROTECH, USA) according to manufacturer's recommendations.

### 2.8. RNA extraction and cDNA synthesis

Total RNA was extracted from the skin fragments (three skin fragments/sample) by mechanical homogenisation with Ultra Turrax in Trizol Reagent according to the manufacturer's protocol (Life Technologies). The amount of total extracted RNA was estimated measuring the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios by Nanodrop (ND-1000 UV–Vis Spectrophotometer; NanoDrop Technologies). The integrity of RNA was evaluated by agarose gel electrophoresis. For each sample, 1000 ng of total RNA were retro-transcribed with iScriptTM cDNA synthesis kit (Bio-Rad), following the manufacturer's instructions.

### 2.9. Gene expression by real-time qPCR

For real-time qPCR experiments, the data from each cDNA sample were normalized using the human housekeeping gene RLP0 (ribosomal protein lateral stalk subunit P0). The specific primers used for amplification of RLP0, IL-6, IL-8, TNF-α and COX-2 were designed based on the nucleotide sequences downloaded by NCBI database using Primer3WEB v.4.0.0 (see supplementary table 1). A final concentration of 1.4 pmol/μl for each primer, 1 μl of 1:10 diluted cDNA having initial concentration of 1 μg and FastStart SYBR Green Master Mix (total volume of 10 μl) were used for the reaction mixture. PCR amplifications were performed in a ViiATM 7 real-time PCR (Applied Biosystems) thermal cycler system using the following thermal profile: 95 °C for 10 min, one cycle for cDNA denaturation; 95 °C for 15 s and 60 °C for 1 min, 40 cycles for amplification; 72 °C for 5 min, one cycle for final elongation; and one cycle for melting curve analysis (from 60 °C to 95 °C) to verify the presence of a single product. Each assay included a negative control missing the cDNA template for each primers pair. Specificity of amplification reactions was verified by melting curve analysis. The efficiency of each primers pair was calculated according to standard method curves using the equation $E = 10^{-1/slope}$. At least three serial dilutions 1:2, 1:10 and 1:100 were set up to determine Ct values and reaction efficiencies for all primer pairs. Standard curves were generated for each oligonucleotide pair using the Ct values versus the logarithm of each dilution. The expression of the genes was analysed and internally normalized against RLP0 using relative expression software tool (REST) software based on the Pfaffl method [48]. Relative expression ratios above two cycles were considered significant. Experiments were repeated at least three times.

### 2.10. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, USA). All data are the results of at least two independent experiments carried out in triplicate. Data were expressed as the mean ± standard deviation. Comparisons among groups were made by Student's *t*-test. Values of $p < 0.05$ were considered as significant.

For RT-qPCR analysis, in REST software the results of the expression ratio of the transcripts are tested for significance by a Pair Wise Fixed Reallocation Randomization Test and plotted using standard error (SE) estimation via a complex Taylor algorithm. Relative expression ratios approaching two cycles were considered significant.

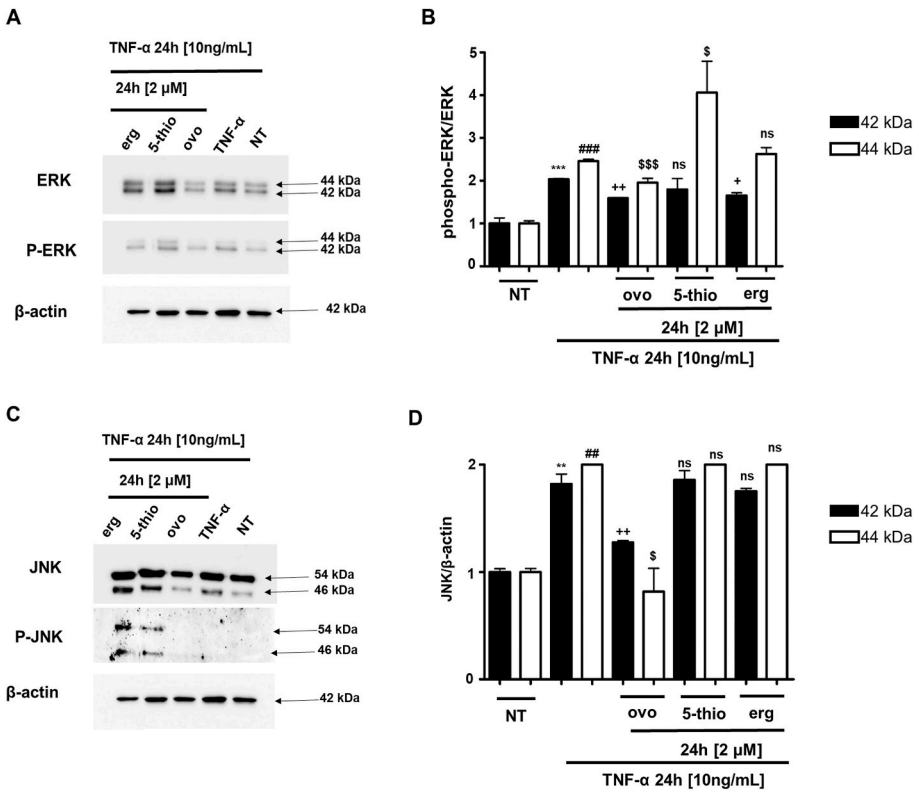


**Fig. 3. Protein expression of MAPKs upon inflammation and thiohistidine pre-treatment (A)** and **(C)** Representative experiments of Western blot analysis of cytosolic extracts obtained from HaCaT cells pre-treated for 24 h with ovo, 5-thio, and erg at concentration of 2 μM, and stimulated for 24 h with TNF-α at 10 ng/ml to induce inflammation. **(B, D)** Histograms of the densitometry analysis of protein bands obtained by Western blot using ImageJ software. **(B)** Data of P-ERK were normalized against ERK. **(D)** Data of JNK were normalized against β-actin. Data are expressed as mean ± SD, $n = 3$, the significance was determined by the *t*-test student. **(B)** *** ($p < 0.001$) represent significance compared to NT band at 42 kDa; ### ($p < 0.001$) represent significance compared to NT band at 44 kDa;+($p < 0.05$), ++ ($p < 0.01$) represent significance compared to TNF-α treatment band at 42 kDa and $ ($p < 0.05$), $ $$ ($p < 0.001$) represent significance compared to TNF-α treatment band at 44 kDa. **(D)** ** ($p < 0.01$) represent significance compared to NT band at 46 kDa; ## ($p < 0.01$) represent significance compared to NT band at 54 kDa; ++ ($p < 0.01$) represent significance compared to TNF-α treatment band at 46 kDa; $ ($p < 0.05$) represent significance compared to TNF-α treatment band.

## 3. Results

### 3.1. Sulfur-containing histidine compounds are not cytotoxic in HaCaT cells

The cytotoxicity of the sulfur-containing histidine compounds ovothiol (ovo), 5-thiohistidine (5-thio) and ergothioneine (erg) (see Fig. 1A for molecular structure) was examined on HaCaT cells. Cell viability was assessed following treatment with ovo, 5-thio and erg at concentrations of 2, 5, 10, 50, and 100 μM for 24, 48 and 72 h. The results showed that ovo, 5-thio, and erg were not cytotoxic in the range of 2–10 μM (Fig. 1B–D), although after 72 h treatment with 5 and 10 μM a slight decrease of cell viability was observed. By contrast, a significant increase in cell viability at the lowest concentration of 2 μM was observed especially at 24 h. On the other hand, a slight but significant decrease in cell viability was monitored especially at 50–100 μM (Fig. 1B–D).

### 3.2. Sulfur-containing histidine compounds protect HaCaT cells against TNF-α-induced cytotoxicity

To investigate if sulfur-containing histidine compounds could protect human keratinocytes against TNF-α-induced cytotoxicity and inflammation, we pre-treated HaCaT cells with ovo, 5-thio, and erg at not cytotoxic concentrations of 2, 5, and 10 μM for 24 h and then we induced inflammation by treating the cells for 24 h with TNF-α at concentrations of 1, 2, 5 and 10 ng/ml. Subsequently, cell viability was assessed by MTT assay, which evaluates the metabolic capacity of living cells. TNF-α-mediated inflammation caused a slight but significant reduction of viability at concentrations of 2, 5, and 10 ng/ml, while having no effect at 1 ng/mL concentration, compared to not treated cells (NT) (Fig. 2A–D). On the other hand, the pre-treatment with ovo, 5-thio, and erg prevented the cells from the decrease in viability caused by TNF-α, especially at 2 and 5 μM (Fig. 2A–D). In particular, all the molecules at 2 μM induced, a more pronounced increase in cell viability in comparison to cells treated with TNF-α alone (Fig. 2B–D).

### 3.3. Effect of sulfur-containing histidine compounds on MAPKs signalling

To investigate whether the pre-treatment with ovo, 5-thio, and erg at the lowest effective concentration (2 μM) could mediate the activation of the MAPKs cascade upon inflammation, we evaluated ERK and JNK phosphorylation by western blot analyses (Fig. 3A–C). The immunepositivity given by p-ERK and p-JNK was normalized against the unphosphorylated forms of ERK and JNK, respectively (Fig. 3A–D).

The protein expression of ERK was detected using an antibody that recognizes two isoforms of the protein (44 and 42 kDa). The expression of both ERK isoforms did not significantly vary in any treated sample, compared to the constitutive β-actin. On the other hand, the densitometric analysis of p-ERK *versus* ERK, showed an increase in intensity of both bands (42 and 44 kDa) when HaCaT cells were treated only with TNF-α compared to NT cells (Fig. 3 B). In cells pre-treated with ovo, we observed a significant decrease in the protein expression of p-ERK for both bands (42 and 44 kDa) compared to cells treated only with TNF-α (Fig. 3 B), however ERK remained activated compared to NT (*p value* = 0.0048 for 42 kDa and *p value* = 0.0018 for 44 kDa). On the other hand, the pre-treatment with 5-thio did not induce any effect on 42 kDa p-ERK form, while inducing a significant increase of the 44 kDa band when compared to the cells treated only with TNF-α (Fig. 3 B). An opposite trend was observed in cells pre-treated with erg, for which we observed a significant decrease of intensity only of the p-ERK 42 kDa band compared to cells treated only with TNF-α (Fig. 3B), although higher compared to NT ($p = 0.0047$).

Furthermore, we evaluated the protein expression of JNK, using β-actin as normalizer (Figure C). In this case, we observed an increase in both isoforms of JNK (46 kDa and 54 kDa) in cells treated with TNF-α alone compared to NT cells (Fig. 3C). The pre-treatment with ovo induced a significant decrease of both bands (46 kDa and 54 kDa) compared to cells upon inflammatory stimulation alone, while the pre-treatment with 5-thio and erg did not induce such change (Fig. 3 D).

Regarding the phosphorylated form of JNK protein, we did not detect any band, neither at 46 kDa or 54 kDa in NT cells as well as in cells

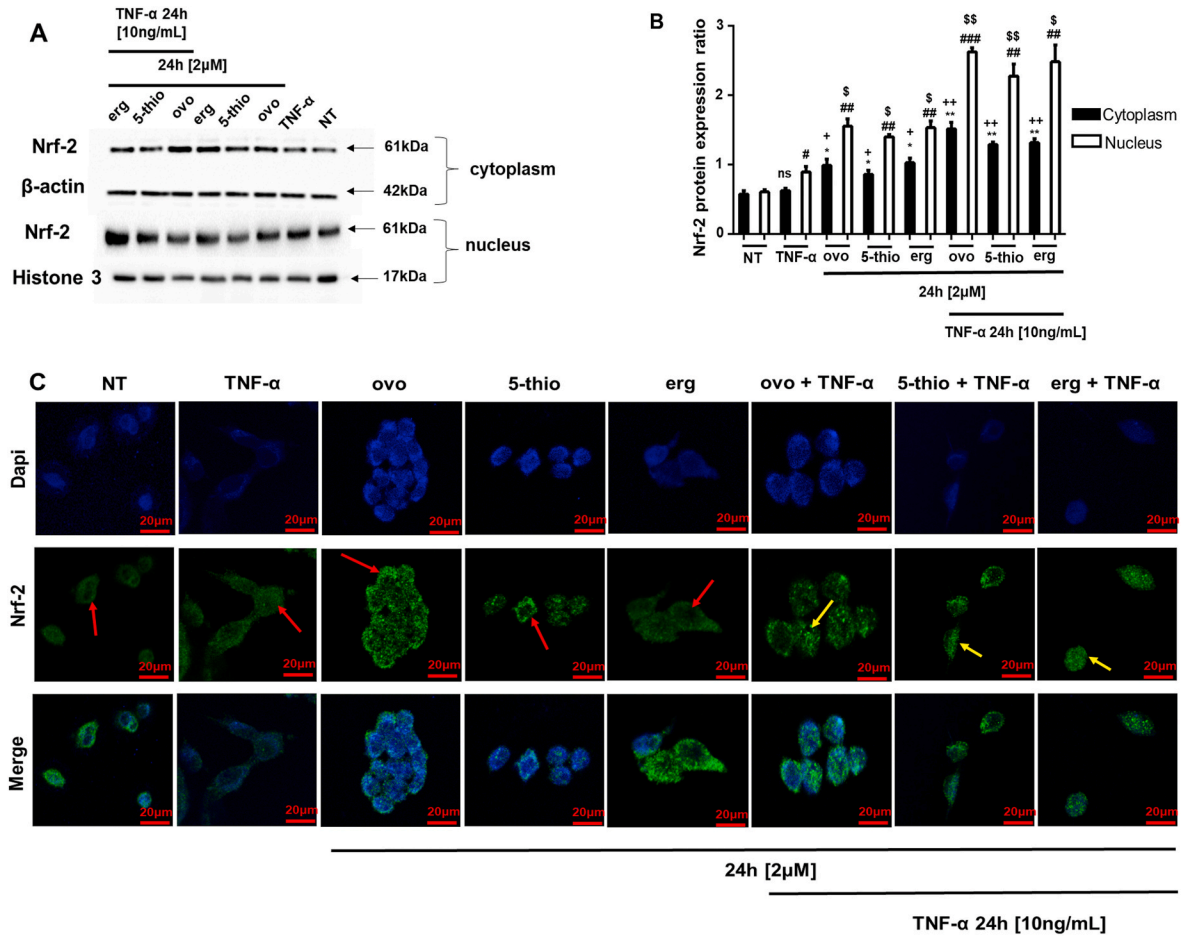


**Fig. 4. Nuclear translocation of NRF2 following sulfur-containing histidine compounds treatment. (A)** Representative experiment of Western blot analysis of cytosolic and nuclear extracts obtained from HaCaT cells pre-treated for 24 h with ovo, 5-thio, and erg at concentration of 2 μM and stimulated for 24 h with TNF-α at 10 ng/ml to induce inflammation. **(B)** Histograms of the densitometry analysis of protein bands obtained by Western blot using ImageJ software. Data were normalized for β-actin (cytosolic fraction) and Histone 3 (nuclear fraction). Data are expressed as mean ± SD, $n = 3$, the significance was determined by the *t*-test student,*($p < 0.05$) and **($p < 0.01$) represent significance compared to NT cytoplasmic fraction; $^{\#}$ ($p < 0.05$), $^{\#\#}$ ($p < 0.01$) and $^{\#\#\#}$ ($p < 0.001$) represent significance compared to NT nuclear fraction;+($p < 0.05$), $^{++}$ ($p < 0.01$) represent significance compared to TNF-α treated cytoplasmic fraction; $^{\$}$ ($p < 0.05$) and $^{\$\$}$ ($p < 0.01$) represent significance compared to TNF-α treated nuclear fraction. **(C)** Immunofluorescence staining showing the changes of Nrf2 fluorescence. DAPI (1 μg/ml) was stained for 10 min and examined by fluorescence microscopy. Scale bars, 20 μm; acquisition 40x by Observer.Z1 Zeiss LSM 700. Red arrows indicate the cytosolic localization of Nrf2 while the yellow arrows represent the localization of nuclear Nrf2.

stimulated only with TNF-α (Fig. 3 D). Also the pre-treatment with ovo did not induce the phosphorylation of JNK, on the contrary the pre-treatment with 5-thio and erg induced the phosphorylation of both bands of JNK (Fig. 3 D). The absence of p-JNK bands both in NT and TNF-α stimulated cells prevented to perform a quantitative densitometric analysis of p-JNK/JNK ratio.

### *3.4. Sulfur-containing histidine compounds promote Nrf2 nuclear translocation in HaCaT cells*

To determine whether the pre-treatment with ovo, 5-thio, and erg could mediate the nuclear translocation of Nrf2, we evaluated both the cytoplasmic and nuclear fractions by western blot using a specific antibody (Fig. 4A–B). In this case, the cytoplasmic fraction was normalized against β-actin, while the nuclear fraction was normalized against histone 3 (Fig. 4A–B). The results showed no significant variation of Nrf2 at the cytoplasmic level in cells treated with TNF-α in comparison to NT (Fig. 4B) while we observed a slight increase for Nrf2 at the nucleus level in cells treated with TNF-α in comparison to NT (Fig. 4B). On the other hand, when HaCaT cells were treated only with sulfur-containing histidine compounds, a significant accumulation of Nrf2 was observed compared both to NT and to cells treated only with TNF-α. The accumulation of Nrf2 was more pronounced in the nucleus compared to the cytoplasm. (Fig. 4B). In cells pre-treated with compounds and then stimulated with TNF-α, we observed a further accumulation of Nrf2 both at the cytoplasmic and nuclear level compared to NT, to cells treated only with TNF-α, and to cells pre-treated only with compounds (Fig. 4B and supplementary table S2-3). However, also in this case, the accumulation of Nrf2 was greater in the nucleus than in cytoplasm.

To further confirm these data, nuclear import of Nrf2 in control and treated cells was monitored by immunofluorescence (Fig. 4C). Nrf2 was mainly tethered in the cytoplasm of NT cells (as indicated by the red arrow in Fig. 4C); while a small amount of nuclear Nrf2 was detected after TNF-α treatment (Fig. 4C). At the same time, in cells treated only with sulfur-containing compounds, Nrf2 appears to be partially tethered in the cytoplasm, but in a less pronounced manner than NT, and partially localized in the nucleus (as indicated by the red arrow in Fig. 4C). Instead, pronounced Nrf2 fluorescence intensity especially in the nucleus was observed in samples pre-treated with ovo, 5-thio, and erg plus TNF-α compared to NT and TNF-α alone (Fig. 4C yellow arrows). These results indicate that sulfur-containing histidine compounds promote Nrf2 nuclear translocation, especially after TNF-α (inflammation) treatment.

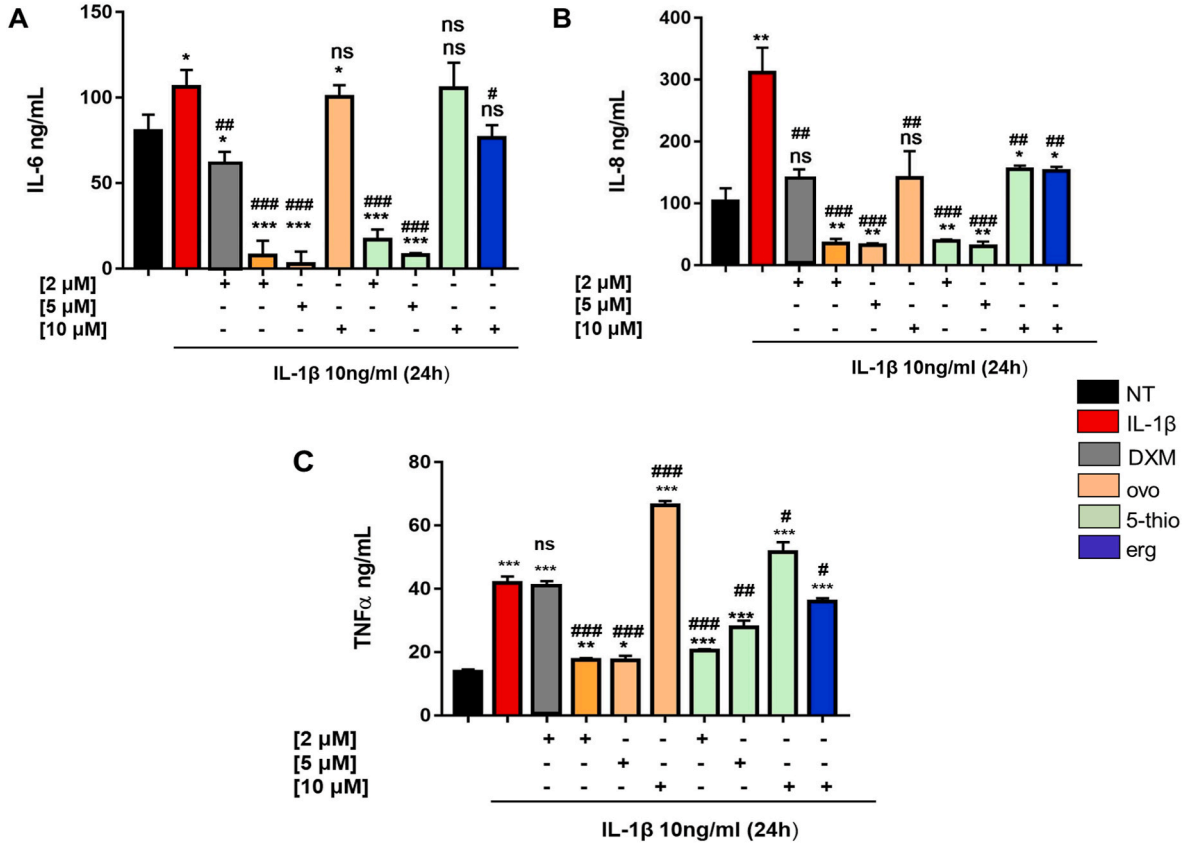


**Fig. 5. Accumulation of pro-inflammatory cytokines in *ex vivo* human skin tissues.** Tissues were pre-treated for 24 h with ovo, 5-thio, and erg at three different concentrations (2, 5 and 10 μM) and inflammation was stimulated for 24 h with IL-1β 10 ng/ml. The release of cytokines IL-6 (**A**), IL-8 (**B**), and TNF-α (**C**) was measured by ELISA assay. Data are expressed as mean ± SD, n = 3. The significance was determined by the *t*-test student and post hoc analysis: * ($p < 0.05$), ** ($p < 0.01$) *** ($p < 0.001$) represent significance compared to NT (not treated only medium) control; # ($p < 0.05$), ## ($p < 0.01$) ### ($p < 0.001$) represent significance compared to IL-1β 10 ng/ml (24 h). In the histogram, data referred to NT cells are in black, to cells after inflammation in red, to cells pretreated with dexamethasone (DXM) in grey, with ovo in orange, with 5-thio in green, with erg in blue.

### *3.5. Anti-inflammatory activity of sulfur-containing histidine compounds on ex vivo human skin tissues*

Since the results described above pointed to a protective activity of sulfur-containing histidine compounds against induced inflammation in HaCaT cells, we further investigated on the protective properties of these molecules on fragments of human skin tissues, also including the keratinocytes layer. To this aim we used ELISA assays to measure the levels of the cytokines IL-6, IL-8 and TNF-α released from *ex-vivo* human skin tissue fragments, pre-treated with the compounds and then stimulated with IL-1β, compared to NT controls. As expected, IL-1β-induced inflammation caused a significant increase in IL-6, IL-8 and TNF-α release compared to NT tissue fragments (Fig. 5A–C). The 24 h pre-treatment with ovo and 5-thio, at the concentration of 2 and 5 μM, led to a drastic decrease of cytokines release, especially for IL-6 and IL-8, thus indicating a strong anti-inflammatory effect. Interestingly, 10 μM concentration was less effective in reducing the release of IL-6 and IL-8, and even induced the release of TNF-α (Fig. 5A–C). Pre-treatment with erg 10 μM resulted in a slight but significant decrease of IL-6 and TNF-α, and a more pronounced decrease of IL-8, following the inflammation stimulus. Interestingly, pre-treatment with ovo and 5-thio was even more effective than the pre-treatment with dexamethasone (DXM), a type of corticosteroid medication used to treat many inflammatory conditions, including skin diseases [47] (Fig. 5A–C).

### *3.6. Sulfur-containing histidine compounds affect gene expression of inflammatory markers in ex vivo human skin tissues*

To evaluate also the modulation of gene expression of the tested cytokines and specific inflammatory markers involved in the progression of human skin damage, RTqPCR analysis of *IL-6*, *IL-8*, *TNF-α*, and *COX-2* was carried out after the pre-treatment of explant skin tissues with 2, 5 and 10 μM of ovo, 5-thio, and erg, followed by inflammatory stimulus of 10 ng/ml IL-1β. As expected, a significant increase in gene expression of *IL-8*, *TNF-α*, and *COX-2* was observed in *ex-vivo* human skin tissues treated with IL-1β alone, compared to NT fragment tissues (reference baseline), while we did not observe a significant increase in gene expression for *IL-6* (Fig. 6A). On the other hand, skin samples pre-treated for 24 h with ovo, 5-thio, and erg showed a significant down-regulation of *IL-8*, *TNF-α* and *COX-2* compared to skin tissues treated with IL-1β alone (reference baseline) at all concentration tested, except for the pre-treatment with erg 10 μM, which did not significantly affect the gene expression of *IL-8* (Fig. 6B–D). On the contrary, *IL-6* resulted to be up-regulated upon the pre-treatment with the molecules at 2 μM, while not displaying significant variations at 5 and 10 μM. Moreover, the pre-treatment with DXM at 2 μM did not significantly affect the gene expression of *IL-6* and *TNF-α*, but significantly induced the down-regulation of *IL-8* and *COX-2* (Fig. 6 E).

## 4. Discussion

Our previous investigations pointed to the anti-inflammatory properties of a new class of marine sulfur-containing amino acid derivatives:

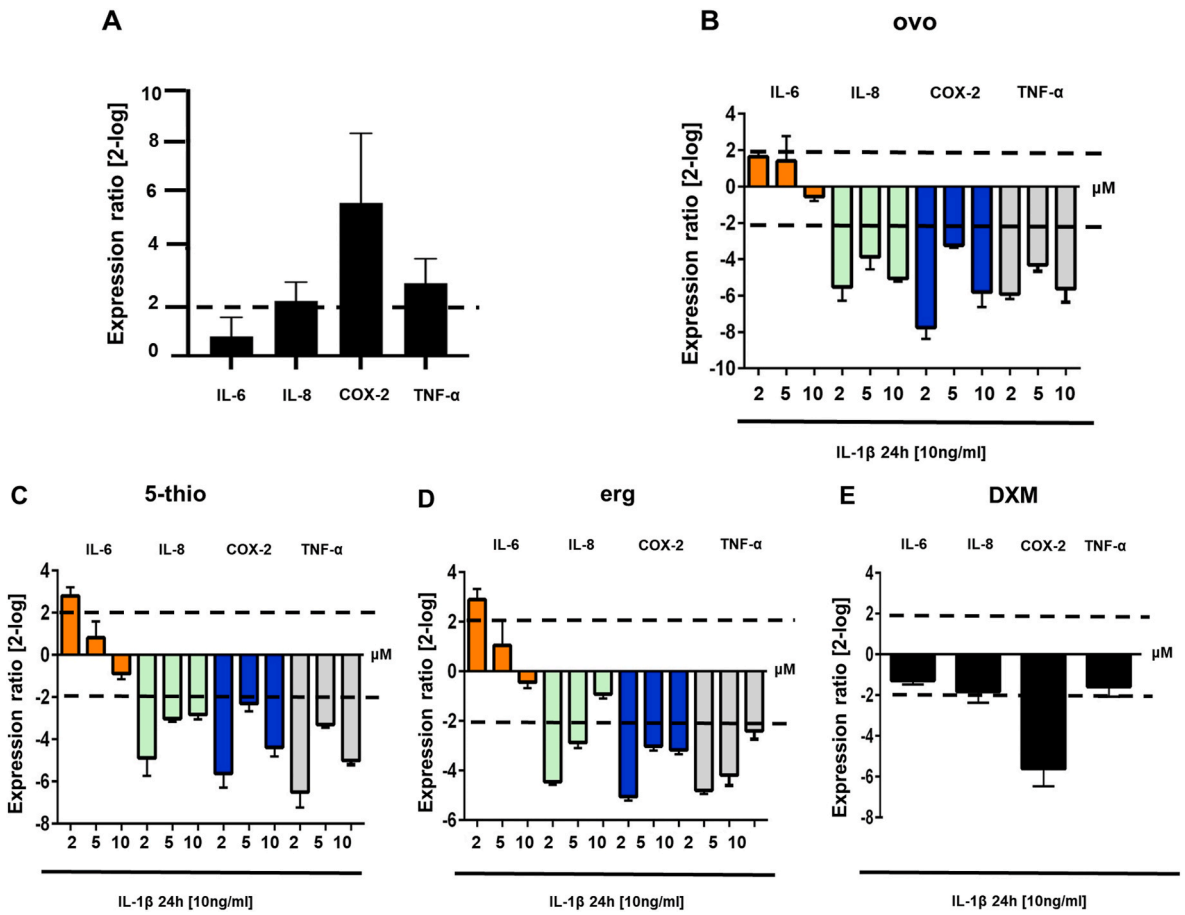


**Fig. 6. Effect of sulfur histidine compounds on gene expression of inflammatory markers in *ex vivo* human skin tissues.** The levels of gene expression of inflammatory markers were assessed in skin tissues pre-treated with, ovo, 5-thio, erg, and DXM as positive control (see figures for concentrations) for 24 h and then treated with IL-1β 10 ng/ml for other 24 h to induce inflammation. **(A)** Tissues treated with only 10 ng/ml IL-1β for 24 h were compared to NT tissues (reference baseline). **(B–E)** Tissues pre-treated with ovo, 5-thio, erg, and DMX up to 24 h and then treated with IL-1β 10 ng/ml for other 24 h were compared to tissues treated with only 10 ng/ml IL-1β for 24 h (reference baseline). Data were analysed through the REST software, relative expression ratios equal or major of the threshold value ± 2 were considered significant.

5-thio and its methylated form, ovo [41–43]. In this work, for the first time, we tested the protective activity of these compounds against the inflammatory processes associated with skin damage, which in the last decade has become a major health problem due to the exacerbation of environmental threats, including air and ocean pollution, global warming and the thinning of the ozone layer [26]. Indeed, the continuous exposure to environmental stressors leads to skin aging and can induce dermal pathologies, whose effects are deleterious, cumulative, and often irreversible. Currently, skin cancer is the most common cancer worldwide [49] and other skin pathologies associated with inflammation affect 20–40% of the population [50–52].

In this study we demonstrated that low doses of marine-derived 5-thiohistidine compounds are able to protect human keratinocytes against the cytotoxic effect induced by TNF-α and to reduce the levels of pro-inflammatory interleukins in human skin tissues stimulated with IL-1β. The positive effects of the different forms of 5-thiohistidine derivatives are likely due to the differential ability to affect ERK or JNK signalling, thus favouring the nuclear translocation/stabilization of Nrf2 and the down regulation of inflammatory genes, like *IL-8*, *TNF-α*, and the inducible form of cyclooxygenase, *COX-2*.

Briefly, the treatment of human keratinocytes with sulfur-containing histidine compounds, did not induce a relevant cytotoxicity, but only a slight decrease in cell vitality at the higher doses of 50–100 μM. Indeed, the observed inverted U-shaped dose response, characterized by a slight increase in proliferation at the lowest concentrations, suggests the occurrence of an adaptive response of the cells, commonly known as hormesis. This phenomenon, characteristic of many drugs and xenobiotics, deserves further investigations.

More interesting was the finding that the pre-treatment at low concentrations of ovo, 5-thio, and erg protected human keratinocytes from the reduced vitality observed upon TNF-α induced inflammation, thus suggesting that the tested compounds exert protective/anti-inflammatory activity on human keratinocytes.

Considering that during an inflammatory process, the MAPK cascade is activated through increased ERK and JNK phosphorylation [14–17], we evaluated the protein expression of ERK and p-ERK, JNK and p-JNK upon treatment with sulfur-containing histidine compounds. The different effect induced by ovo, 5-thio, and erg pre-treatment on the two ERK forms, ERK1 (p44) and ERK2 (p42), undoubtedly suggests a different mechanism of action for such molecules. It is likely that the different chemical and structural properties of such compounds may account for their different activity behaviour. Indeed, while ovo and 5-thio are administrated in their more stable disulfide form and differ only for a methylation on the imidazole group of histidine, erg is characterized by a thiol group in position 2 of the imidazole ring and is stabilized in thionic form in physiological conditions [46]. Actually, ovo is a more efficient scavenger of radicals and peroxides compared to erg, while the methylation on the imidazole group of histidine in ovo may stabilize the resonance forms of the thiolate compared to 5-thio [33]. Since during inflammation the production of ROS generally increases, together with the activation of MAPKs, leading to a dysregulation of the skin homeostasis, it is very likely that ovo reacts with ROS more actively

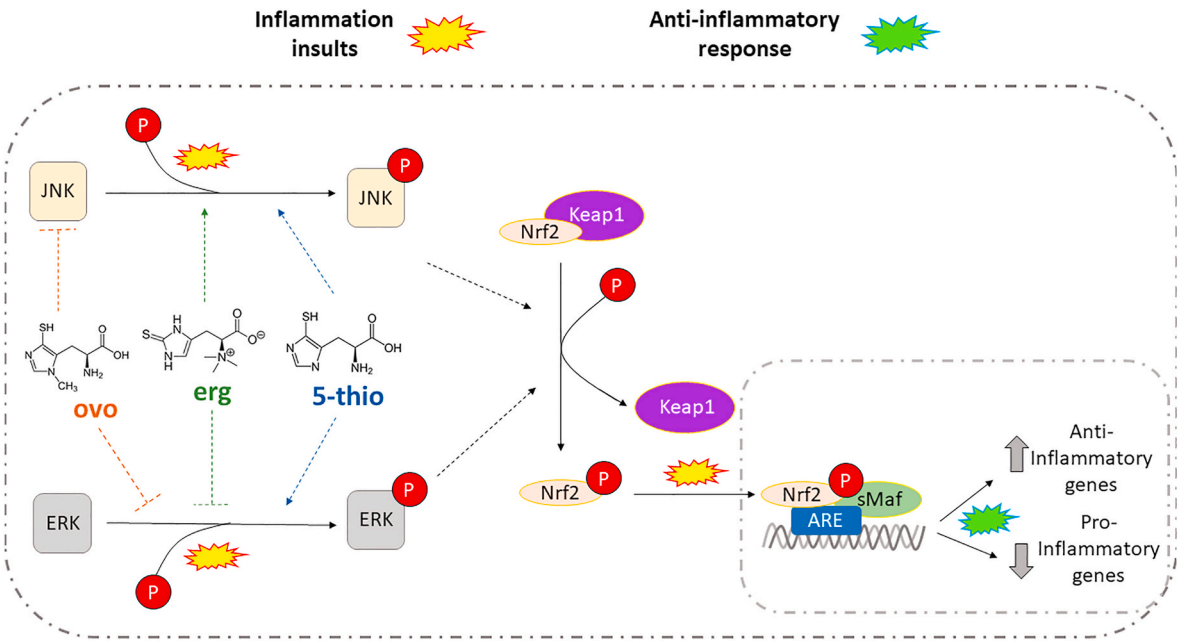


**Fig. 7. Proposed mechanism of action for thio-histidine compounds**. Treatment with ovo, before inflammation of the skin tissue, prevents ERK 1/2 phosphorylation and JNK accumulation. Treatment with 5-thio, before inflammation of the skin tissue, promotes ERK 2 and JNK phosphorylation. Treatment with erg prevents ERK 1 phosphorylation and promotes JNK phosphorylation. These events mediate the activation of Nrf2, which migrates into the nucleus and interacts with the ARE sequences, thus leading to the down-regulation of COX-2 and inhibiting the release of the pro-inflammatory cytokines.

compared to 5-thio and erg, thus inducing the decrease of ERK phosphorylation and contributing to restore skin homeostasis. The involvement of JNK resulted to be more complex to explain; the observed increase in proteins for both JNK isoforms (46 and 54 kDa) upon induction of cell inflammation, compared to the decrease of the protein content when the cells were pre-treated with ovo, suggests that ovo may act on the regulation of JNK biosynthesis/degradation. This finding is also supported by the failing in detecting any band for JNK phosphorylation, after ovo pre-treatment. On the other hand, the pre-treatment with 5-thio and erg did not induce any effect on JNK protein content compared to the treatment only with TNF-α, while inducing the phosphorylation of both JNK forms. These results indicate that ovo may negatively affect JNK, that is instead activated by 5-thio and erg. Thus, our finding suggests that ovo affects MAPKs signalling by acting mainly on ERK phosphorylation likely through ROS scavenging, whereas 5-thio and erg act mainly through JNK phosphorylation.

Considering that MAPKs activation is essential for cell survival following exposure to stress conditions, and that it can induce the phosphorylation of Nrf2 and subsequently its translocation to the nucleus to prompt the transcription of antioxidant genes [20], the finding that the compounds tested in this study promote the accumulation of Nrf2 in the nucleus, indicates that ERK and JNK activation may mediate this process.

It is likely that when keratinocytes are stimulated with TNF-α, Nrf2 is activated in response to stress signals, thus inducing its translocation to the nucleus to prompt transcriptional activity. Actually, we do not know if the activation and accumulation of Nrf2 in the nucleus in response to stress signals is due to its stabilization or to the prevention of its degradation [20]. However, electrophiles and ROS are known to interact and oxidize reduced cysteine residues in Keap1 causing the dissociation of Keap1 from Nrf2, and its consequent activation [53]. It has been also shown that Keap1 can sense hydrogen peroxide by formation of disulphide bridges [54] and can undergo S-glutathionylation [55], thus losing the ability to target Nrf2 for ubiquitination, and therefore promoting the newly synthetized Nrf2 accumulation [56]. These findings are particularly interesting as sulfur-containing histidine might react with cysteine residues of Keap1 forming disulfide bonds, and disrupting the association with Nrf2, thus promoting its translocation to the nucleus. Indeed, many electrophilic natural compounds, able to react with thiols, like sulforaphane have been demonstrated to be Nrf2 inducers [57]. Actually, we do not know exactly if our compounds induce the translocation of Nrf2 through redox regulation of Keap1 or MAPK signalling, or by a cross-talk between these two mechanisms. It is likely that the activation of p-ERK via TNF-α is sufficient for the dissociation of cytoplasmic Nrf2/Keap1 and the subsequent Nrf2 nuclear import and maybe the pre-treatment with our compounds can promote this process or protect Nrf2 from degradation through cysteine oxidation. Indeed, ovo pre-treatment prevented ERK over-phosphorylation, while sustaining ERK activation at sufficient levels (compared to physiological conditions) to prompt Nrf2 accumulation into the nucleus. However, ovo is also known to make redox exchange with glutathione [58] and thus it is very likely that it can also form disulfide bond with cysteine residues [33].

In order to further confirm the anti-inflammatory activity of sulfur-histidine compounds in a more physiological environment, we tested their activity also in *ex vivo* human skin tissues collected from patients undergoing cosmetic surgery. We found that, the pre-treatment of ovo, 5-thio, and erg induced the down-regulation of *IL-8*, *TNF-α* and *COX-2* genes, normally up-regulated upon inflammation, together with the inhibition of pro-inflammatory cytokines release. The finding that the 10 μM treatment with ovo and 5-thio was less effective compared to 2 and 5 μM, suggests that even lower concentrations can be able to protect skin tissue against inflammation.

Overall, the findings that thiohistidines promote the accumulation of Nrf2 in the nucleus of keratinocytes and simultaneously prevent the release of interleukins, and induce the downregulation of pro-inflammatory genes in human skin tissues, further confirm that these compounds exhibit a protective activity against inflammation. More importantly, the dermo-protective activity of such compounds resulted more efficient compared to the approved topical corticosteroid DXM, thus highlighting competitive advantages for their therapeutic use.

## 5. Conclusions

Overall marine-derived sulfur-containing histidine compounds prevent the release of pro-inflammatory cytokines (IL-6, IL8 and TNF-α) in human skin tissues exposed to inflammation. We propose the following mechanism of action for such compounds (Fig. 7). The administration of ovo, before inflammation stimulus, sustains ERK 1/2 phosphorylation and prevents JNK activation by acting on the biosynthesis/degradation of the protein. On the other hand, the treatment with 5-thio and erg, before inflammatory stimuli, slightly affects ERK2 phosphorylation while promoting JNK phosphorylation. These different mechanisms of action, likely together with redox exchange mechanisms between thiols favour the activation of Nrf2, which interacting into the nucleus with the antioxidant responsive elements sequences, leads to the downregulation of inflammatory genes such as COX-2.

Considering that Nrf2 represents one of the master transcriptional regulators of the antioxidant and anti-inflammatory response [59], also preventing cell death [60], and that Nrf2 activators represent a pharmacological strategy against inflammatory conditions associated with the exposure to emerging environmental stressors [61], marine derived 5-thiohistidines can be considered promising molecules to protect the skin against pro-inflammatory external stimuli. Consequently, their molecular structure can inspire the development of novel and eco-safe products for skin care with protective and anti-inflammatory activities.

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## Author contributions

MB, DS and IC conceived and designed the experiments. AM, CV, MB performed the experiments. AM, IC, CV, DS, AP analyzed the data. DS and IC provided funds. MB and IC drafted the article. All authors revised and approved the final version of the manuscript.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.09.017.

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