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Nrf2 Pathway Involvement in the Beneficial Skin Effects of Moderate Ionic Osmotic Stress—The Case of The Dead Sea Water

Dror Cohen[1,2], Ze'evi Ma'or[2,3], Meital Portugal Cohen[2,4], Miriam Oron[5], Ron Kohen[1*]

[1]The Myers Skin Research Laboratory, Faculty of Medicine, Institute for Drug Research, School of Pharmacy, Hebrew University of Jerusalem, Jerusalem, Israel
[2]The Skin Research Institute, The Dead-Sea & Arava Science Center, Masada, Israel
[3]Fosun Jinmei (Shanghai) Cosmetics Co., Ltd., Shanghai, China
[4]AHAVA Dead Sea Laboratories, Lod, Israel
[5]Miriam Oron Mingelgrin Consulting, Jerusalem, Israel
Email: drorc@ahava.com, Zeevi.jm@yuyuantm.com.cn, Meital@ahava.com, *ronk@ekmd.huji.ac.il

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Abstract

Objectives: Exposing skin to moderate ionic osmotic stress (MIOS) triggers several biochemical responses. The objective of this work is to reveal the mechanism triggered by MIOS on the skin surface. Furthermore, this work aims to study the involvement of the Nrf2 (nuclear factor erythroid-2-related factor 2) pathway, activated by MIOS, and its beneficial effect in protecting skin against stress via the stimulation of phase II enzymes. Methods: HaCaT cells and human skin organ culture were exposed to Dead Sea Water (DSW) as MIOS inducers and the induction of internal ROS elevation, Nrf2 translocation, mRNA gene expressions of the phase II enzymes, heme-oxygenase 1 (HO1), and Catalase (CAT) were determined. Results: Skin exposure to MIOS increases Nrf2 translocation to the nucleus, leading to increased levels of ROS, HO1, and CAT. Furthermore, exposing skin to MIOS promotes protection against UVB-related risks. This is demonstrated by attenuation of the expression of biomarkers, related to UVB-induced damage, Caspase-3, IL-8, and IL-1β. Conclusions: Skin exposure to MIOS leads to the activation of Nrf2 skin defense pathway and, therefore, could present beneficial advantages to human skin health, as demonstrated on human skin models. The beneficial effects of MIOS, induced by DSW are significantly superior to eq. NaCl brine, suggests that MIOS protection of skin against stress is partially related to specific mineral combinations.

Keywords

Nrf2 Pathway, Ionic Osmotic Stress, Dead Sea Water, Oxidative Stress,

Human Skin Model

1. Introduction

Various types of environmental stressors affect our skin's health [1] [2] [3]. When exogenous interferences "sensed" stress, physiological response is triggered [1] [3] [4]. Among the various stressors, moderate osmotic stress is known for its generally positive effect on skin cells [5]. It has been widely used for health and beauty treatments in ancient times and is still very popular today [6] [7]. Osmotic stress is induced on skin in various forms, including topical mineral treatments of muds, salts and brines [8] [9]. Indeed, ions (mono or multivalent) are involved in many skin biological activities [10] [11] [12]. Dissolved mineral salts act through the induction of cell osmosis mechanism. This is sensed by a mechano-transduction reaction via piezo-electric ion channels [9]. Moderate ionic osmotic stress (MIOS) was reported to have beneficial contributions to skin health, such as enhancing the epidermal barrier functioning, elevating skin's hydration, and reducing the inflammatory response in several skin diseases [13] [14]. When hypersaline solutions are applied on skin, MIOS affects the modulation of cell-cycle dynamics. This is evident in reduced expression levels of inflammatory cytokines and in altered cell proliferation and differentiation rates [13] [15]. Specific external ion levels, mainly magnesium, calcium, potassium, and bromide, have been shown to affect cell proliferation and differentiation [13] [15] [16] [17] [18].

Mineral treatments are widely prescribed in dermatology, formulated in cosmetic products and applied in topical spa treatments [19] [20]. MIOS exposure is one of the proposed biological mechanisms behind the claimed skin therapeutic effects of minerals [7] [10] [11]. A particular case of MIOS's beneficial effect on human skin, is the topical exposure to Dead Sea minerals. The Dead Sea contains high levels of magnesium, calcium, and bromide ions and is clinically proven for its beneficial effects on several skin diseases [15] [21] [22]. Although these effects have been thoroughly studied on patients with skin diseases, the mechanism of MIOS involved as a key generating step for clinical efficacy, is not fully understood. Stressors usually result in endogenous production of reactive oxygen species (ROS) [1] [18] [23]. The level of the induced stress, the duration of the exposure and the type of cells exposed, determine whether the outcome is deleterious or beneficial. This is most probably the case, when skin is exposed to MIOS, which may eventually lead to ROS generation within skin cells [24]. This affects the physiological redox homeostasis of the skin and evokes the induction of various biochemical pathways [25] [26] [27]. The defense mechanism of skin, as in the case of other physiological tissues, against any kind of stress, involves the induction of the phase II enzymes. This group of proteins is mainly regulated by the Nrf2 (nuclear factor erythroid-2-related factor 2) and involved in the detoxification of a wide variety of chemicals and xenobiotics. Phase II enzymes are

involved in the modulation of skin's immune system, thus protecting from inflammatory process. Nrf2 is considered "the master cytoprotective transcription factor" in the cell, responsible for regulating the physiological redox homeostasis. Maintaining cellular redox balance is one of the major roles of phase II proteins [28]. This group comprises the familiar antioxidant enzymes (superoxide dismutase, Catalase (CAT), peroxidase, NAD(P)H dehydrogenase [quinone], heme oxygenase 1 (HO1), glutathione reductase, etc.). Different environmental stressors, including high temperature [29], solar radiation [24] [30], heavy metals [31], and briny water with high mineral concentration [32], were reported to activate Nrf2 in skin cells. The Nrf2 biological pathway is known for its major role in protecting and maintaining skin cells' homeostasis and coping with stress conditions, such as osmotic stress [32]. Hence, in this study, we hypothesized that exposure to MIOS could be beneficial to skin health via the involvement of Nrf2 pathway. We argue that MIOS skin exposure, such as in the case of the Dead Sea water, would result in enhancing the capability of skin to cope with exogenous insults, such as UV irradiation.

2. Methods

2.1. HaCaT Cell Culture

Immortalized non-tumorigenic human epidermal keratinocyte cells (HaCaT), purchased from CLS Cell Lines Service GmbH, (Eppelheim, Germany), were grown in Dulbecco's Modified Eagle's medium (DMEM) (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Biological Industries, Beit Haemek, Israel). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C, at 60% - 70% confluency.

2.2. Human Skin Organ Culture (HSOC)

Human skin organ culture (HSOC) models for biological tests on epidermis fragments were obtained from 20- to 60-year-old healthy women, who had undergone breast or abdomen reduction, in accordance with the Declaration of Helsinki and the Hadassah University Hospital Ethics Committee approval (#0639-12-HMO). Fresh Human skin was cut within 24 hours from donation, to approximately 0.16 cm^2 pieces, cultured with their epidermal side facing up in a petri dish containing DMEM, 100 U/ml penicillin, and 100 U/ml streptomycin and incubated at 37˚C, 5% CO_2 for 24 hours. Epidermis was separated from dermis by 1-min heating to 56˚C in phosphate-buffered saline (PBS). The remaining culture media was collected for cytokines level detection.

2.3. MIOS Exposure/Treatment

Dead Sea water (DSW) was collected on July 2020 from Dead Sea lake's southern basin, in coordinate's latitude, longitude: 31˚1'17.99"N, 35˚22'3.03". HSOC treatments (3 µl, DSW, or 3 µl NaCl 6.15M) were topically applied to the upper

air-exposed epidermis. HaCaT cells were treated with either a final concentration of 1% DSW or 100 mM NaCl in culture medium.

For DSW, the total cationic charge equivalent molarity was calculated by doubling the cation charge (2+/+1) with its concentration in mol/L (see Table 1). The final cation equivalent concentration for DSW was calculated by: (3.91M × 2) + (0.95M × 2) + (0.11M × 1) + (0.03 × 1) + (0.01M × 2) = 9.9M.

NaCl salt was used as a reference for monovalent cationic charged MIOS-compared to the DSW MIOS. HSOC explants were exposed to MIOS by topical treatment with 100% DSW solution or saturated solution of NaCl (6.15M). The detailed DSW ion composition is described in Table 1.

2.4. Apoptosis Determination by Caspase-3 Activity Assay

As described in Portugal-Cohen *et al.* (2009) [33]. In brief, epidermis samples were incubated in 100 µl PBS containing 2.5 lm Ac-DEVD-AMC as a caspase-3 substrate, with 0.02% Triton X-100 and 10 mm DTT, at 37˚C in a 96-well plate. Fluorescence of the released coumarin derivative was measured at 390/435 nm, using a Fluoroskan™ Microplate (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. Composition and osmolarity (Osm/L) of DSW and NaCl solutions. Figures of the elements in the chart are given in M concentrations.

Name	Symbol	Conc. M (mol/L)			
		DSW		NaCl	
		DSW as is	1% solution	Saturated solution	0.10M
Sodium	Na^+	0.11	0.001	6.15	0.10
Potassium	K^+	0.03	0.000	-	-
Calcium	Ca^{2+}	0.95	0.009	-	-
Magnesium	Mg^{2+}	3.91	0.039	-	-
Strontium	Sr^{2+}	0.01	0.000	-	-
Chloride	Cl^-	9.87	0.001	6.15	0.10
Bromide	Br^-	0.13	-	-	-
Total osmolarity of all ions		15.0	0.15	12.3	0.20
Total Cations					
Osmolarity Cations		5.5	0.05	6.15	0.10
Total Charge Cations osmolarity		9.9	0.10	6.15	0.10
Total Anions					
Osmolarity Anions		10	0.10	6.15	0.10
Total Charge Anions osmolarity		10	0.10	6.15	0.10

The activity was given by the fluorescence-versus-time slope, calculated over 30 min in the linear range.

2.5. ROS Analysis

ROS generation in the cells was measured in a plate-reader using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) [34]. HaCaT cells were seeded at 70% - 80% confluence (~50,000 cells/well) in 96-well plates loaded with 100 µM H_2DCFDA (Invitrogen, Carlsbad, California, USA) and DMEM for 30 minutes. Cells were washed 3 times with PBS and incubated with applied MIOS for 30 min at 37˚C. The plates were read by a fluorescence plate reader at Excitation/Emission of 485/530 nm, using a fluorescence reader Fluoroskan™ Microplate (Thermo Fisher Scientific, Waltham, MA, USA). Each assay was performed in 6 replicates at least three times. HSOC explants were incubated with topical treatment of MIOS for 1 hour. The epidermis sheets were separated from the dermis as mentioned above and incubated with 100 µM H_2DCF in DMEM for 30 min at 37˚C. Then, they were washed with PBS and extracted in DMSO and measured at Excitation/Emission of 485/530 nm.

2.6. Mitochondrial Membrane Potential Assay (JC-1)

Mitochondrial depolarization was evaluated using a mitochondrial membrane potential ($\Delta\Psi_M$) kit (Cayman Chemicals, Ann Arbor, MI, USA) with the incorporation of JC-1 (5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolcarbocyanineiodide) [34]. Briefly, HaCaT Cells were seeded at 1.5×10^4 cells/well in 96 wells black culture plate and the day after treated with 1% (*v*/*v*) DSW, or 100 mM NaCl in the culture medium. After 20 hours, a 10 µM solution of JC-1 in DMEM culture medium was added to each well and incubated for 30 minutes. Cells were gently washed 3 times with PBS and maintained in the designated assay buffer. Mitochondrial activity was calculated as the ratio between Excitation/Emission wavelengths 535/595 nm and 485/533 nm and presented as % relative to the mean values detected from the positive control.

2.7. Intracellular Ca^{2+} Measurement Assay

As described in Pulli *et al.* (2014) [35]. In brief intracellular Ca^{2+} concentration was measured with the [Ca^{2+}]i indicator dye 1-[2-(5-carboxyoxal-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2'-amino-5'-methylphenoxy-ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (Fura-2) (Abcam, Cambridge, MA, USA)). HaCaT cells were seeded at a density of 1.5×10^4 cells/well in a 96-well plate. The following day cells were loaded with a 5 µM Fura-2 solution, diluted in Ringer buffer solution: 20 mM HEPES pH-7.4, 150 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose and incubated for 30 minutes at 37˚C under 5% CO_2. Cells were washed twice with PBS buffer and thereafter treated with ionic osmotic stress for one hour. Intercellular Ca^{2+} was calculated as the ratio between the Excitation/Emission wavelengths 335/505 nm and 363/512 nm and presented as %

relative to the mean values detected from the positive control. Fluorescence was monitored in an interval of 1 hour to 4 hours.

2.8. Nrf2 Pathway Activation Assessment by Real-Time PCR

RNA was extracted from HaCaT cells using Triazole reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. HSOC epidermis was homogenized using the RNeasy mini kit according to the manufacturer's protocol (QIAGEN, Venlo, Netherlands). A reverse transcription kit (Invitrogen, Carlsbad, California, USA) was used to construct the template cDNA and the resulting cDNA products were diluted in a final volume of 60 µl. Quantitative real-time PCR was performed in a 10 µl reaction mixture containing 5 µl SyberGreen low ROX (Invitrogen, Carlsbad, California, USA), 0.1 µM of each primer, 2.8 µl H_2O, and 2 µl of template cDNA working solution. Products were amplified with human heme oxygenase 1 (HO1) primers (forward: 5'-GGC AGA GAA TGC TGA GTT CAT GAG GA-3' and reverse: 5'-ATA GAT GTG GTA CAG GGA GGC CAT CA-3'), Catalase (CAT) primers (forward: 5'-ATG TGC ATG CAG GAC AAT CAG GGT-3', and reverse: 5'-AAT ATT GGA TGC TGT GCT CCA GGG-3'). In all cases, the samples were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (forward: 5'-TCG ACA GTC AGC CGC ATC TTC TTT-3' and reverse: 5'-ACC AAA TCC GTT GAC TCC GAC CTT-3'). Amplifications were performed using a Thermo Real-Time PCR Detection System (Thermo, Waltham, MA, USA). Each assay was performed in triplicate at least three times.

2.9. Translocation of Nrf2 and Distribution of HO1/Nrf2 Assessment by Immunofluorescence

HaCaT cells were grown in 96-well plates with a glass-bottom (Greiner, Kremsmünster, Austria)] and exposed overnight to 1% (*v*/*v*) DSW, 100 mM NaCl or 0.1 mM *tert*-Butylhydroquinone (tBHQ) as an inducer of the transcription of Nrf2-dependent genes [36]. Ochratoxin A was used as Nrf2 translocation inhibitor [37] [38] [39] [40] [41]. Cells were fixed in 4% paraformaldehyde immediately after the appropriate treatment period was finalized. HSOC explants were topical treatment with 3 µl of DSW and incubated for 2, 6 and 24 hours. After incubation, skin explants were fixed with paraffin-embedded and cut into 10 µm-thick sections. Following deparaffinization and rehydration in a series of xylenes-ethanol washes, sections were permeabilized with 0.25% Triton/PBS and blocked with 1% (*v*/*v*) bovine serum albumin (BSA) in PBS. Samples were then incubated with the primary anti-Nrf2 antibody (ab62352, Abcam, Cambridge, UK) in 1% (*v*/*v*) BSA/PBS, or anti-HO1 antibody (ab13248, Abcam, Cambridge, UK), followed by a secondary anti-rabbit FITC-((Sigma-Aldrich, St. Louis, MO, USA) or anti-rabbit Alexa Fluor®647 (111-605-045, Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) antibodies. Finally, sections were mounted with anti-fade solution (1% (*w*/*v*) N-propyl-gallate (MP Biomedical, Santa Ana, CA, USA) in 0.1M Tris buffer in glycerol). Fluorescence visualization

was carried out in a CYTATION-3 reader (BioTek, Winooski, VT USA). The fluorescence signal was quantified by ImageJ NIH software (NIH, Washington, DC, USA). Nrf2 translocation was expressed as the ratio of the fluorescent signal detected within the nucleus vs. cytoplasm [42] [43] [44].

2.10. UVB Irradiation of Human Skin Organ Culture

Prior to UVB irradiation, the culture medium was discarded, and the skin explants were washed in PBS to remove all culture medium. Skin explants were placed in PBS while the epidermis remained exposed to air. Samples were irradiated with a UVB artificial source (VL-6.M lamp, UVB mediumwave emission spectrum 280 - 350 nm, emission peak 312 nm, Spectroline, New York, USA) at a sub-lethal dose of 200 Mj/cm^2. Immediately after irradiation, PBS was replaced by DMEM growth medium, and skin explants were further incubated for various periods of time.

2.11. Evaluation of Cytokines Secretion

IL-1β and IL-8 levels were assayed by a "sandwich" ELISA kit (BioLegend, San Diego, CA, USA) as described in Portugal-Cohen *et al.* (2009) [33]. Briefly, HSOC media were collected after incubation and stored at −80˚C. A 96 wells plate was used to incubate standards and samples with the precoated immobilized antibodies. After immune binding of the antigen, a second enzyme-linked, monoclonal or polyclonal antibody directed to the same antigen, was added. After completion of a "sandwich" formation, unbound molecules were washed away, and a colorogenic TMB substrate was added to reveal the enzyme-linked antibody. The enzymatic reaction was stopped with 0.5M H_2SO_4. The plate was read at 450 nm O.D using a Fluoroskan™ Microplate (Thermo Fisher Scientific, Waltham, MA, USA). Cytokine concentrations were calculated by comparison with standard solutions.

2.12. Statistical Analysis

Each experiment was performed at least in triplicate. Average values are given with standard error of the mean (SEM). Differences between average values were tested for significance using the unpaired Student t-test and considered as significant for $P < 0.05$.

3. Results

3.1. Skin Exposure to MIOS Induces the Activation of Nrf2 Cellular Protective Pathway

3.1.1. Nrf2 Translocation into the Nucleus in HaCaT Cells and HSOC Skin Explants, Following Their Exposure to MIOS

The ratio between Nrf2 protein in the nucleus and Nrf2 in the cytoplasm was quantified, following exposure of skin cells to MIOS, to monitor the first essential step in the activation of Nrf2 pathway, its translocation to the cell nucleus [42] [45]. HaCaT cells were stained with Nrf2 antibodies following exposure to

1% (*v*/*v*) DSW, 100 mM NaCl or 0.1 mM *tert*-Butylhydroquinone (tBHQ) to demonstrate Nrf2 intracellular translocation. The ratio between the fluorescence signals obtained from cell's nucleus and cell's cytoplasm was used to determine Nrf2 nucleus translocation level. Cells incubated with 0.1 mM tBHQ, used as a positive control, presenting an increase in the nucleus/cytoplasm Nrf2 signal ratio of 2.15 compared to the untreated control of 1.5 (**Figure 1(a)**). In comparison, HaCaT cells exposed to MIOS either by 1% (*v*/*v*) DSW or 100 mM NaCl, showed nucleus/cytoplasm ratio of 2.01 and 1.98, respectively.

The localization and translocation of the Nrf2 protein were also evaluated in HSOC skin explants exposed to MIOS in the form of DSW. The nucleus/cytoplasm signals ratio was significantly higher by 8% after 2 hours and 5% after 6 hours compared with the untreated epidermis. This effect vanished after 24 hours of exposure and returned to the ratio of the untreated control (**Figure 1(b)**).

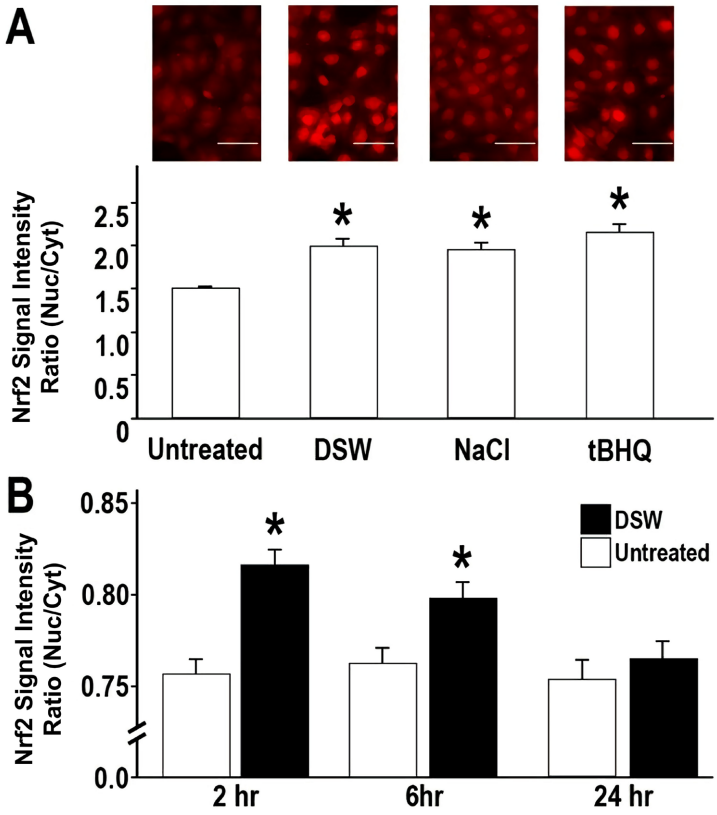


Figure 1. Temporal localization of Nrf2 following DSW exposure. (A) 6 hours exposure of HaCaT cells to either 1% (*v*/*v*) DSW, 100 mM NaCl or 0.1 mM tBHQ. The upper part represents images of HaCaT cells stained for Nrf2 (white scale bar eq. to 100 μm); the lower part, quantitative analysis calculating the ratio between the signal intensity detected from each cell's nucleus signal to its cytoplasm signal. (B) HSOC explants were topically exposed to MIOS by applying a layer of 19 μl/cm^2 of 100% DSW solution for 2, 6, and 24 hours. Nrf2 translocation was detected using confocal microscopy and immunofluorescent staining of the cells/HSOC with Alexa 647 fluorescent dye. Results show the ratio between the signal intensity detected from each cell's nucleus signal to its cytoplasm area after ImageJ processing. The values are expressed as the mean ± SE. *$p < 0.001$ vs. untreated cells ($n > 90$)

3.1.2. Transcription Upregulation of Phase II Enzymes, Heme-Oxygenase 1 (HO1) and CAT, Following Skin Exposure to MIOS

Phase II enzymes, known for their general protecting properties against stressors, were monitored using the RT PCR method. mRNA levels of two crucial phase II genes were measured, Heme-oxygenase 1 (HO-1) and Catalase (CAT). Figure 2 shows the gene expression of the two cutaneous phase-II antioxidant enzymes following exposure to MIOS, both in skin cells and in *ex-vivo* skin explants. HaCaT skin cell culture and HSOC explants were exposed to MIOS of either DSW or NaCl. Quantitative mRNA levels were measured and normalized to the expression levels of the housekeeping gene, *gapdh*. A significant elevation of HO1 mRNA expression level by 2.1 and 1.6 folds compared to the untreated control is evident in HaCaT cells, following exposure to MIOS of 1% DSW or 100 mM NaCl respectively (Figure 2(a)). In order to further elucidate the involvement of the Nrf2-pathway in the observed phase II enzymes induction, Ochratoxin A (OTA), an inhibitor of Nrf2, was used., [37] [38] [39] [40] [41]. OTA with 1% DSW prevented the MIOS elevation of HO1 expression in HaCaT cells. CAT mRNA levels were not affected following the exposure of the HaCaT cells to DSW MIOS with or without OTA in cell media (Figure 2(a)).

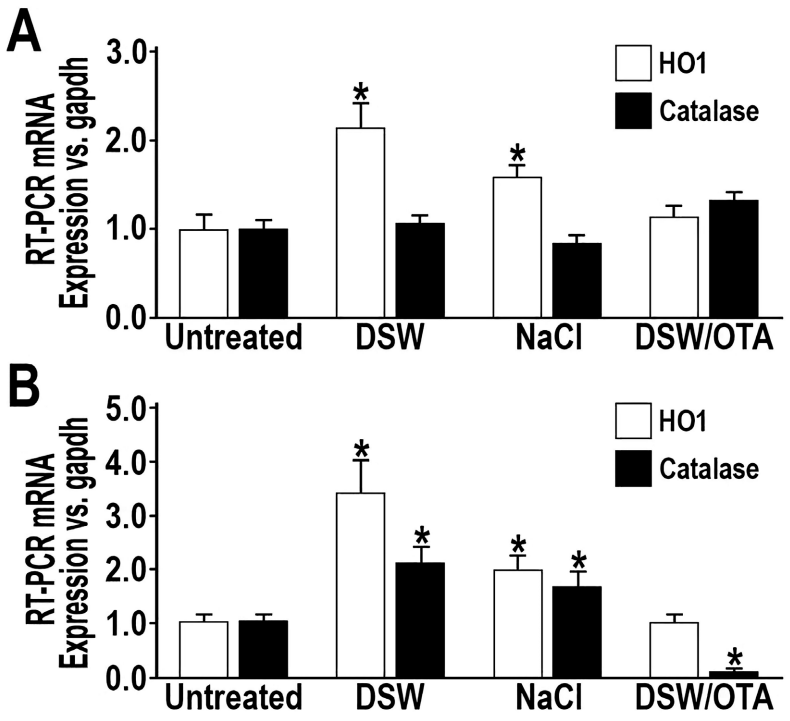


Figure 2. The mRNA Expression level of HO1 (white bars) or CAT (black bars) on HaCaT cells (A) *ex-vivo* HSOC (B) following MIOS exposure. HaCaT cells were exposed to 1% (*v*/*v*) DSW, 100 mM NaCl or 1% (*v*/*v*) DSW, with or without 25 µM OTA and incubated for 6 hours. HSOC explants were topically applied with either 19 µl/cm^2 of 6.15M NaCl or 100% of DSW, with or without 3.1 µM OTA and incubated for 48 hours. The expression levels of HO1 and CAT mRNA were determined by RT-PCR, and results were normalized according to the expression of the housekeeping gene, *gapdh*. *$p < 0.05$ vs. correlated control. Data are presented as mean ± SE.

HSOC explants were exposed to MIOS by topical application of 100% DSW or a saturated (6.15M) NaCl solution. The mRNA expression level of HO1 was significantly increased by 3.4 folds, following the exposure to DSW, and by 2.0 folds with NaCl compared to the untreated control. After skin exposure to MIOS of DSW and NaCl, the mRNA expression levels of CAT were increased by 2.1 and 1.7 folds, respectively, compared to the untreated control. Adding OTA, to DSW prevented the MIOS effect. Interestingly, a significant reduction of CAT mRNA level was measured following OTA addition to the media before topical application of DSW compared to the untreated control (**Figure 2(b)**).

3.2. Elucidation of the Mechanism of Nrf2 Induction, Following Skin Exposure to MIOS

3.2.1. Elevated Endogenous ROS Level in HaCaT Cells and Skin Organ Culture HSOC Induced by MIOS

ROS production, monitored via the fluorescent probe H_2DCFDA was measured following the exposure of skin cells and skin explants to MIOS [46]. HaCaT cells loaded with H_2DCFDA were exposed to MIOS by adding 1% (*v*/*v*) DSW or 100

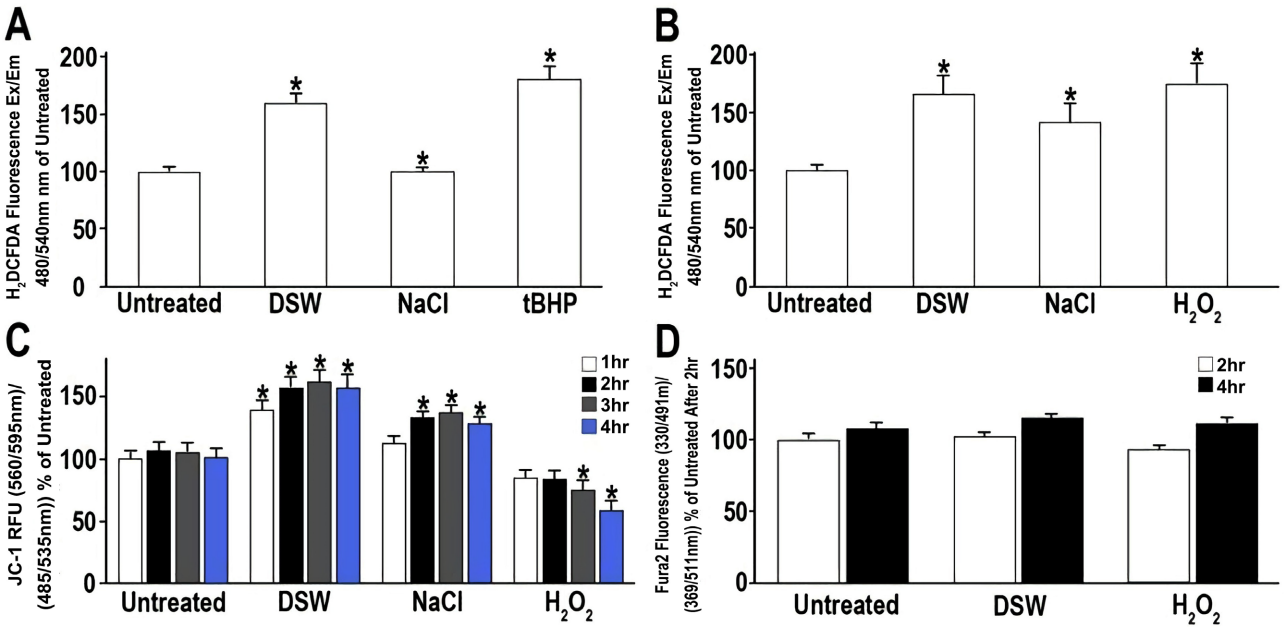


Figure 3. Mechanism aspects of Nrf2 induction following skin exposure to MIOS. (A) Endogenous ROS generation after the exposure of HaCaT cells to MIOS. HaCaT cells were incubated for 30 min in a culture medium containing 1% (*v*/*v*) DSW, 100 mM NaCl, or 0.1 mM tBHP. (B) Endogenous ROS generation after HSOC explants were topically exposed to DSW and NaCl MIOS for 30 min. HSOC explants were topically applied with 25 µl/cm^2 of 100% DSW solution, 6.15M NaCl, or 5 mM H_2O_2, and then epidermis was separated from the dermis and incubated with H_2DCFDA for 30 min. Intracellular ROS levels were determined by fluorescence intensity, measured at Ex/Em 485/530 nm. (C) ΔΨm following HaCaT cells exposure to MIOS. HaCaT cells exposure to MIOS of 1% (*v*/*v*) DSW, 100 mM NaCl, or 0.1mM H_2O_2. HaCaT cells were loaded with JC-1 probe, monitored after 1 - 4 hours. The ΔΨm was calculated by the fluorescence intensity ratio of (560/595 nm) to (485/535 nm). (D) Intracellular [Ca^{2+}] level in the HaCaT cells. HaCaT cells were incubated for 20 minutes with Fura-2. Cells were exposed to 1% (*v*/*v*) DSW, 100 mM NaCl or 0.1mM H_2O_2 for 2 hours (white bars) and 4 hours (black bars). Intracellular [Ca^{2+}] levels were determined from Fluorescence intensity (Ex/Em, 340/380 nm) measurements. All values are presented as the mean ± SE. Significance, *p < 0.05, vs. correlated untreated control.

mM NaCl to the culture media. HaCaT cells were exposed to 0.1 mM *tert*-Butyl-hydroperoxide (tBHP) that served as a positive control. The cell's incubation with 1% (*v*/*v*) DSW, resulted in a significant elevation of ROS levels by 1.6 folds in comparison to untreated cells. After treatment with tBHP, ROS levels were elevated by 1.8 folds compared to untreated cells, while 100 mM NaCl did not significantly elevate ROS levels (**Figure 3(a)**).

Skin exposure to MIOS on HSOC explants was performed by topical application of either 100% DSW or 6.15M NaCl solution. HSOC exposed to DSW topical MIOS increased the fluorescent signal of H_2DCFDA by 1.7 folds and 6.15M NaCl by 1.4 fold, in comparison to the untreated skin. A 1.8-fold increase was measured when skin was exposed to H_2O_2, the positive control (**Figure 3(b)**). Both DSW and NaCl induced a significant elevation of ROS levels compared to the untreated control.

3.2.2. Increase in HaCaT Mitochondrial Membrane Potential (ΔΨm) Following Exposure to MIOS

Since the mitochondria are the main source of ROS production in the cell, mitochondrial membrane potential (ΔΨm) was measured in HaCaT cells, using JC-1, a probe for ΔΨm [34]. Following cells exposure to DSW MIOS for 20 hours, the ΔΨm was significantly increased compared to both untreated control and H_2O_2 exposed cells. A similar less intense trend was obtained when MIOS was induced by incubating HaCaT cells with 100 mM of NaCl in culture medium (**Figure 3(c)**).

3.2.3. Endogenous Ca^{2+} Levels in HaCaT Cells Are Not Altered by MIOS

The intracellular level of calcium ions was measured to assess its sensitivity to changes in ROS levels. Calcium cations [Ca^{2+}] are secondary messengers involved in intra- and extracellular signaling cascades, including those involving the generation of ROS [47] [48]. The endogenous [Ca^{2+}]i level was determined by Fura-2 acetoxymethyl ester (Fura-2), following the exposure of HaCaT cells to MIOS by adding 1% (*v*/*v*) DSW, or 0.1 mM H_2O_2 to the culture medium. No change in the [Ca^{2+}] endogenous level was detected in all of the treatments (**Figure 3(d)**).

3.3. The Protective Effect of MIOS against UVB Induced Inflammation in Skin Explants

Topical application of 6 μl/cm^2 of 100% DSW solution or 6.15M NaCl on HSOC during four consecutive days followed by UVB irradiation was set as a model for UV-induced damage under repeated MIOS exposure. Skin viability of untreated and treated HSOC was similarly reduced following MIOS exposure (data not shown). Untreated UV-irradiated HSOC shows upregulation of Caspase-3, an apoptosis-related enzyme (**Figure 4(a)**). The exposure of HSOC to DSW for four consecutive days followed by UVB irradiation resulted in decreased activity of Caspase-3 by 2.13 folds, compared to the irradiated epidermis control. Topical exposure to a 6.15M NaCl solution showed increased caspase-3 activity by 1.6

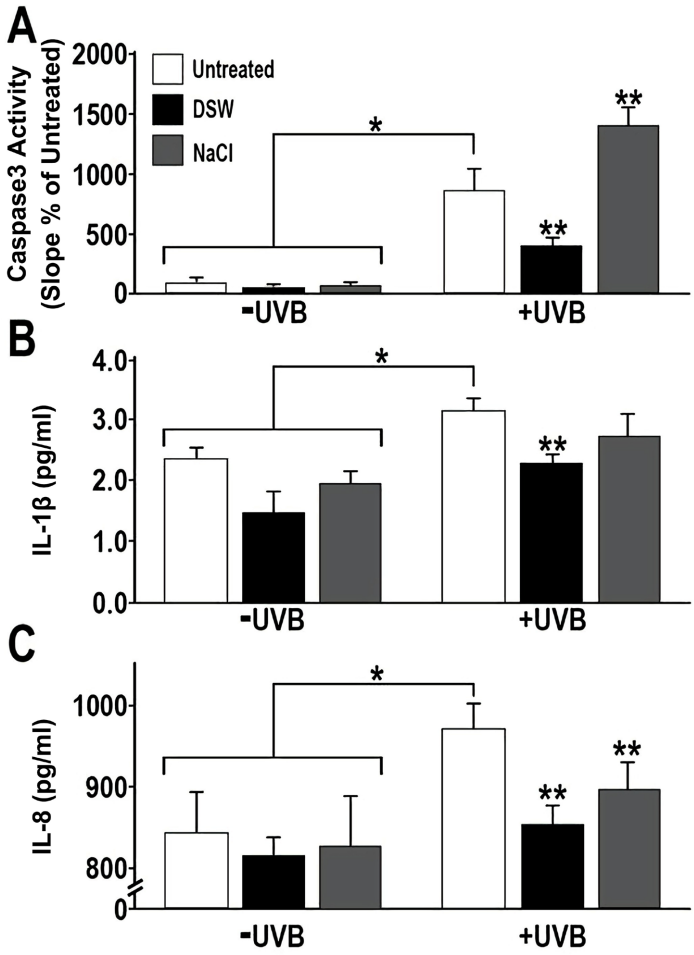


Figure 4. The protective effect of HSOC pre-exposure to MIOS when followed by a high dose of UVB radiation. HSOC explants were exposed to topical MIOS of either 9 μl/cm^2 of 100% DSW (black bars) or 6.15M NaCl (grey bars) solution or untreated (white bars) for four consecutive days. After incubation, the HSOC was exposed to UVB irradiation of 200 mJ/cm^2, incubated for 24 hours and the expression levels of relevant inflammation biomarkers were measured. (A) Epidermal apoptosis-related caspase-3 enzymatic activity. Data are presented as % of the untreated control. (B) Cytokine IL-1β secretion to the medium after 24 hours from irradiation. (C) Cytokine IL-8 secretion to the medium after 24 hours from irradiation. Data are presented as pg/ml. *$p < 0.05$ vs. no UVB irradiated samples. **$p < 0.05$ vs. UVB irradiated untreated. The values represent the mean ± SE.

folds compared to the irradiated epidermis control (Figure 4(a)). Skin exposure to DSW MIOS during four consecutive days and after exposure to UVB showed significantly reduced IL-1β and IL-8 secretion levels by 14% and 30%, respectively, compared to the UVB irradiated untreated control. The skin's exposure to 6.15M NaCl solution resulted in a similar effect of reduced IL-1β (not significant) and IL-8 levels by 7.8% and 13%, respectively (Figure 4(b), Figure 4(c)) compared to UVB irradiated untreated control.

4. Discussion

The beneficial impact of minerals on skin has been known for hundreds of years

[49]. However, the interactions between minerals and skin are only partially understood. In this study, we hypothesize that the moderate osmotic pressure, generated by the collection of dissolved and ionized minerals, when applied on skin surface, can induce the defense mechanism of skin, governed by the Nrf2 pathway. The key involvement of this pathway as the vital defensive mechanism to cope with skin's environmental stressors was demonstrated in many studies [50] [51]. Nrf2 is an essential biological pathway, explaining the resilience of skin, continuously affected by various types of outdoor stressors, such as UV radiation, ozone, and environmental pollutants [1] [4] [50] [51] [52]. Moreover, the involvement of Nrf2 transcription factor and subsequent expression of phase II protecting enzymes in skin, as a response to MIOS, has not been fully elucidated.

Translocation of the Nrf2 transcription factor into the nucleus was described as a triggering step for cell's protection, through activation of the Nrf2 pathway [53]. The activation of this pathway includes four stages: translocation, gene expression, protein synthesis and activity of the proteins [54]. Demonstration of the translocation step, though elemental, is not sufficient for claiming activation of this pathway. Quantification of gene expression and protein activities is essential for elucidating the mechanism.

Nrf2 translocation to the nucleus was demonstrated in HaCaT and HSOC following exposure to DSW, as well as in HaCaT cells exposed to NaCl (**Figure 1**). Gene expression of two important phase-II enzymes, HO1 and CAT, were studied to understand the mode of action of Nrf2, following skin exposure to DSW MIOS. HO1 enzyme cleaves the heme ring of Hemoglobin into biliverdin, carbon monoxide, and ferrous ion [55]. This reaction transforms a highly reactive ROS generation molecule, such as heme, into an anti-oxidative character molecule. Exposure of HaCaT skin cells and HSOC to MIOS, exhibited an elevated level of HO1 mRNA. The significantly higher HO1 levels when MIOS was induced by DSW, compared with NaCl, as presented in **Figure 2(a)** and **Figure 2(b)**, suggest the MIOS induced effect on skin is governed by the specific chemical composition of the minerals mixture. The same trend is evident in HSOC with CAT, but not in HaCaT cells. These results are in line with our assumption that the beneficial effects of MIOS on skin are partially dependent on specific ion compositions. Ochratoxin A (OTA) treated HaCaT cells and skin explants, combined with exposure to DSW MIOS, resulted in a significant decrease in CAT activity only in skin explants and no significant change in OH1 mRNA level, compared to the untreated control. Since OTA is an inhibitor of the Nrf2 translocation to the cell nucleus [37] [38] [39] [40] [41], it can be concluded that the Nrf2 pathway is indeed involved in the induction of HO1 mRNA following skin exposure to MIOS in both cell and organ skin models, as described in **Figure 2(a)** and **Figure 2(b)**.

CAT enzyme catalyzes the decomposition of hydrogen peroxide into water and oxygen. This enzymatic activity is an essential cell detoxifying machinery, protecting the cells from excessive levels of hydrogen peroxide, and other ROS-

related oxidative damage [56]. Although HaCaT cells, exposed to MIOS, did not show any attenuation on mRNA transcription level of CAT, the topical exposure of HSOC skin explant model to DSW MIOS showed a superior elevated CAT mRNA level than with MIOS with NaCl. Since the molarity of the two solutions was similar, the difference in osmotic pressure can be attributed to the different identities of its ions. The two model systems demonstrated different results regarding CAT mRNA levels. These differences could be explained as an outcome of the different biological responses of cell culture and the full dermis-epidermis skin model. We can conclude that the elevation of CAT in the skin explant model is connected to the Nrf2 pathway. These results are in line with reported findings that soaking in the Dead Sea water raised the CAT enzymatic level in the epidermis of vitiligo patients [57]. Therefore, we may suggest that the HSOC full skin model, better predicts the *in-vivo* skin response to osmotic stress.

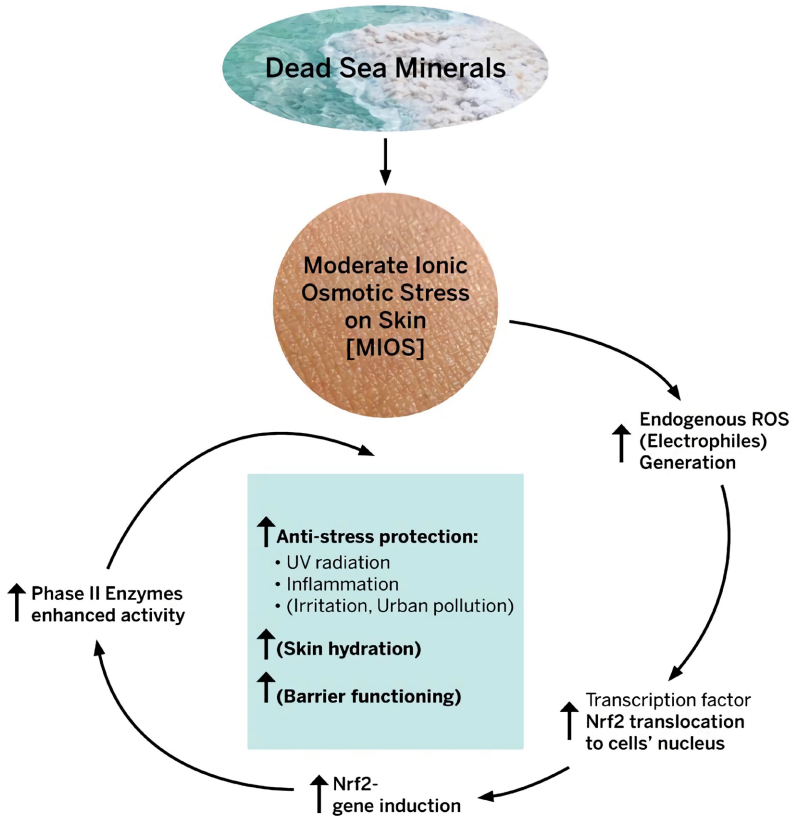
Skin exposure to moderate stress could activate innate protective mechanisms [1]. Our findings, presented in **Figure 4**, show a reduced cytokine's expression levels of interleukins IL-1β and IL-8, and a lower level of caspase-3 activity, following skin irradiation with UVB, subsequent to its earlier exposure to DSW MIOS. Skin's superior resistance to UVB damage, partially supports our assumption of a beneficial protective effect, induced by prior exposure to MIOS. This reported protective effect, is considered to have resulted *via* the activation of Nrf2 mechanism. This assumption is in line with previous reports on improved skin resilience to stress, induced by UV, inflammation, irritation, and urban pollution, following its exposure to DSW [58].

Nrf2 and NFκB are two key transcription factors, regulating cellular responses to oxidative stress and inflammation, with cross-talk between each other [59]. It is expected that induction of the Nrf2 protecting pathway, is associated with a decreased activity of the NFκB. Several studies have proposed functional cross-talk between these two key regulators [60] with Keap1 involvement [61] [62] [63]. It has been proven that Nrf2 depletion, enhances the inflammatory process through the activation of NFκB pathway [59] [64]. Moreover, a variety of anti-inflammatory phytochemicals have been shown to suppress NFκB signaling and activate the Nrf2 pathway [65]. NFκB up-regulation leads to an increased expression of inflammation-related biomarkers, such as interleukins IL-β, IL-8 and Caspase-3. Our results indicate a reduced expression of NFκB related inflammatory proteins, namely cytokines IL-1β and IL-8 and the apoptotic enzyme Caspase-3, following exposure to DSW, as shown in **Figure 4**. Hence, they are in line with the expected downregulation of the NFκB, in parallel to up-regulated Nrf2 transcription factors, and could partially support the functional cross-talk hypothesis of NFκB and Nrf2.

A possible explanation for the observed Nfr2 nucleus translocation could be the intracellular production of electrophiles, such as ROS, following the exposure to MIOS. Indeed, **Figure 3(a)** and **Figure 3(b)** demonstrates the endogenous production of ROS. Therefore, it can be speculated that MIOS is "sensed" by the skin as a kind of stress or threat, leading to endogenous ROS production and

other electrophiles [1] [32]. As shown previously, in many cases, the induction of cellular ROS leads to oxidation of the thiol group on the Keap1 protein, allowing the Nrf2 to translocate into the nucleus [66]. Calcium ions [Ca^{2+}] and ROS are two cross-talking messengers in various cellular processes [67] [68]. Elevation of intracellular [Ca^{2+}] level is involved in the activation of ROS-generating enzymes in the mitochondria respiratory chain [67] [69]. We observed ROS elevation, following the exposure of skin explants to MIOS, but no change in intracellular [Ca^{2+}] level, as shown in **Figure 3(d)**. These findings are in line with Masaki *et al.*, showing that although intracellular [Ca^{2+}] leads to elevation of internal ROS levels, exposure to external ROS does not elevate intracellular [Ca^{2+}] [70].

Mitochondria are the cell's primary internal source of reactive oxygen species (ROS), generated mainly as a by-product of mitochondrial respiratory process at the electron transport chain [71]. Since osmotic stress leads to cellular oxidative stress, the mitochondrial membrane potential is an important indicator of ROS production [25]. Elevation of ($\Delta\Psi m$) has been shown to increase the rate of ROS generation [72]. As shown in **Figure 3(c)**, elevated ($\Delta\Psi m$) following exposure to osmotic stress occurred, accompanied by an increased level of ROS within the cells. These results support the suggested link between MIOS, mitochondrial ROS production, and the activation of Nrf2 pathway.



Scheme 1. A proposed mechanism of mineral-rich Dead Sea skin treatments. Based on our previous work, speculation factors and effects are listed in brackets [58].

In summary, the results, presented in this study, hypothesize that exposure to moderate stress, such as MIOS, is beneficial to skin health, by activating the Nrf2 protective mechanism against insults. Such MIOS may be the consequence of skin exposure to the high concentration of dissolved minerals, naturally existing in the Dead Sea water, as presented in Scheme 1. The Dead Sea minerals are responsible for the moderate osmotic stress, applied to skin, which in turn activates mitochondrial ROS endogenous production, and perhaps other electrophiles [32] [73]. Therefore, we may conclude that skin exposure to osmotic pressure (or stress) leads to the activation of skin cellular defense pathway and, could present beneficial advantages to skin health. The proposed mechanism, presented in this study for the first time, contributes to a better understanding of the cellular biology process, beyond Dead Sea skin beneficial effects, previously established in clinical studies. This study shows differences in the Nrf2 pathway activation by MIOS in skin when using different mineral compositions to induce stress. Further research is needed to establish MIOS proposed mechanism involvement in other beneficial effects on skin such as skin barrier functioning and skin hydration.

It can be speculated that co-exposure to other factors, prevailing in the Dead Sea area, such as high oxygen density, unique sun radiation with exceptional UVA/UVB ratio, and high content of bromine in the air, could additionally contribute to providing skin protection.

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Authors' Contributions Statement

I declare that each one of the co-authors listed in the manuscript entitled "Dead Sea minerals: new findings on skin and the biology beyond" has significantly contributed to its preparation and approval of the final manuscript.

- D. Cohen's contribution was by performing and leading the laboratory research and writing the manuscript;
- Z. Ma'or's contribution was by assisting in writing the manuscript;
- M. Portugal-Cohen's contribution was by assisting in writing the manuscript;
- M. Oron contribution was by assisting in writing the manuscript;
- Ron Kohen's contribution was by supervising the laboratory research and assisting in writing the manuscript.

Conflicts of Interest

The authors certify that they have no affiliations with or involvement in any or-

ganization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Meital Portugal-Cohen is the VP of Science and Technology of AHAVA, the Dead Sea Laboratories.

Dr. Ze'evi Ma'or is the Co-Chief Science Officer, Fosun Cosmetics Group, Head of Jinmei Excellence Innovation Center, Dead Sea Hub.

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