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ITALIAN JOURNAL OF

DERMATOLOGY *and* VENEREOLOGY

OFFICIAL JOURNAL OF THE SOCIETÀ ITALIANA DI DERMATOLOGIA MEDICA, CHIRURGICA, ESTETICA E DELLE MALATTIE SESSUALMENTE TRASMESSE (SIDeMaST)

PUBBLICAZIONE PERIODICA BIMESTRALE - POSTE ITALIANE S.P.A. - SPED IN A.P. D.L. 353/2003 (CONV. IN L. 27/02/2004 N° 46) ART. 1, COMMA 1, DCB/CN - ISSN 2784-8671 TAXE PERÇUE

Società Italiana di Dermatologia
(SIDeMaST)

VOLUME 157 - No. 4 - AUGUST 2022

EDIZIONI MINERVA MEDICA

Online version at https://www.minervamedica.it

Italian Journal of Dermatology and Venereology 2022 August;157(4):335-41
DOI: 10.23736/S2784-8671.22.07251-6

ORIGINAL ARTICLE

Beneficial effects of Rocchetta® oligomineral water in HaCaT keratinocytes after ultraviolet-B irradiation

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ABSTRACT

BACKGROUND: Several reports have previously suggested that oligomineral water may have a beneficial immunomodulatory role in skin physiology. However, molecular, and cellular mechanisms through which oligo-elements act in cutaneous trophism have not yet been fully clarified. Among the external stimuli that affect the skin, ultraviolet (UV) radiation, which is frequently encountered in everyday life, is a major environmental factor of skin damage. Keratinocytes are the major target of UV, and they play a key role in a first line of body defenses. Accumulating evidence suggests that UVB irradiation induces nuclear DNA damage, membrane destruction, resulting in apoptosis and skin inflammation. The aim of this study was to investigate the anti-inflammatory, antioxidant, antiapoptotic effects of Rocchetta® oligomineral (Co.Ge.Di. International SpA, Rome, Italy) water in UVB-irradiated immortalized human keratinocytes.

METHODS: HaCaT UVB-irradiated was cultured with increasing concentrations of Rocchetta® oligomineral water. To evaluate the anti-inflammatory properties gene expression of *TNF, IL1β, IL6, COX2* and *Caspase1* was performed. Moreover, the antiapoptotic effects were evaluated through gene expression of *GADD45*, *Caspase3* and *RIPK3*. Finally, we evaluated the antioxidant activity of Rocchetta® oligomineral water by measuring total ROS/RNS and superoxide production as markers of oxidative stress after UVB irradiation.

RESULTS: Our findings have shown that Rocchetta® oligomineral water is well tolerated by the cells and displays anti-inflammatory, antioxidant and antiapoptotic proprieties when used prior keratinocyte UVB irradiation.

CONCLUSIONS: Our results highlight a possible protective role of Rocchetta oligomineral water in modulating the cutaneous inflammatory response to external triggers and injuries.

(*Cite this article as*: Caiazzo G, Parisi M, Luciano MA, Di Caprio R, Gallo L, Cacciapuoti S, *et al*. Beneficial effects of Rocchetta® oligomineral water in HaCaT keratinocytes after ultraviolet-B irradiation. Ital J Dermatol Venereol 2022;157:335-41. DOI: 10.23736/S2784-8671.22.07251-6)

KEY WORDS: Drinking water; Keratinocytes; Ultraviolet therapy; Anti-inflammatory agents; Antioxidants.

Natural thermal waters have been widely used in the treatment of several inflammatory skin conditions to enhance the healing process.[1] In addition to its therapeutic effect, water plays a key role also as nutritional factor due to its mineral content. Mineral elements, such as sodium, calcium, etc. play a key role in maintaining physiological homeostasis of the body.[2] The term "mineral" refers to all those sources of water with therapeutic properties that are mediated by the type and quantity of the elements that are present. The term "oligo" indicates the small quantity of substances present in the water chemical composition. In 1933, Marotta *et al*.[3] classified mineral waters according to three parameters: temperature, fixed residue, and chemical composition. In particular, fixed residue indicates the number of mineral salts that are dissolved in the water. A fixed residue lower than 200 mg/L defines the low mineral content water, which is the most widely diffused water for nutritional purposes.[4] Rocchetta® oligomineral water (Co.Ge.Di. International SpA, Rome, Italy), differs from thermal waters due to its low concentration of salts (Table I). Therefore, thanks to its low concentration of salts Rocchetta® can quickly enter the circulation, penetrating through the intercellular spaces, that get purified from salts and metabolic refuses (*e.g.*, urea, uric acid) through so-

TABLE I.—*Physical/ chemical parameters of Rocchetta® oligomineral water.*

Parameters	Rocchetta® oligomineral water
pH (Silos)	7.7
Concentration of hydrogen ions in the silos	2×10^{-8} g.ioni/L
Conductivity el. spec. A 20 °C	307.7 µS/cm
Fixed residue −180 °C	184.6 mg/L
Oxidisability	<1.2 mg/L
Free carbon dioxide at the source	6.11 mg/L
Silica	5.042 mg/L
Bicarbonates	190.0 mg/L
Chlorides	7.714 mg/L
Sulphates	7.862 mg/L
Sodium	4.657 mg/L
Potassium	0.356 mg/L
Calcium	59.38 mg/L
Magnesium	4.396 mg/L
Dissolved iron	Not detectable and in any case <0.013 mg/L
Ammonium ion	Not detectable and in any case <0.1 mg/L
Total phosphorus	Not detectable and in any case <0.05 mg/L
Sulfhydrimetric degree	Not detectable and in any case <0.1 mg/L
Strontium	0.1297 mg/L
Lithium	Not detectable and in any case <0.001 mg/L
Aluminum	Not detectable and in any case <0.02 mg/L
Bromides	Not detectable and in any case <0.1 mg/L
Iodides	Not detectable and in any case <0.01 mg/L

dium recall.[5] Water diffusion inside tissues improves intra/extra-cellular exchanges and creates a more favorable environment for cellular metabolism. This, in turn promotes normal organ functions, which in the case of the skin is to provide a physical, biochemical, and immunological barrier to external insults.[5] The skin barrier prevents water loss, the entry of toxic xenobiotics and provides defense against microbial pathogens, physical injuries as well as oxidative stress.[6, 7] During oxidative stress, excessive reactive oxygen species (ROS) overwhelm biological antioxidant capacity, leading to disruption of ROS homeostasis and cell damage.[8] Inflammatory skin diseases, such as psoriasis and atopic dermatitis are associated to an imbalanced cellular metabolism due to oxidative stress.[9-15] Thus, the presence of certain ions at low concentrations in mineral water may have a beneficial effect on cutaneous cell biology, contributing to the restoration of homeostatic metabolism.[16] Several in vitro studies have highlighted the anti-inflammatory, antioxidant, UVB-protective, antiallergic, and antiangiogenic properties of water.[17, 18] For example, spa spring water from Yong-gung in Korea has been shown to reduce the expression of TLR-induced proinflammatory cytokines in HaCaT cells and antigen presenting cell, and on the modulation of CD4+ T cell differentiation.[19] Moreover, a cosmetic preparation containing spa water has been reported to decrease the migratory proprieties of human Langerhans cells.[20] However, despite several reports suggest that oligo-elements play a key role in skin physiology, especially after damage and/or inflammatory insult, their function in cutaneous trophism has not yet been fully clarified. Thus, further research is needed to elucidate the molecular and cellular mechanism underpinning the beneficial immunomodulatory role of mineral water. The aim of this study was to investigate the anti-inflammatory, antioxidant, antiapoptotic effects of Rocchetta® oligomineral water in UVB-irradiated immortalized human keratinocytes (HaCaT). In particular, HaCaT cells were chosen, as they have been shown not only to respond similar to primary skin keratinocytes[21] but also because they lack of interindividual variability.[22]

Materials and methods

Cell culture

Immortalized human keratinocytes, HaCaT cells supplied by CEINGE Biotecnologie Avanzate S.C.R.L. (Naples, Italy), were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO), 2 mM L-glutamine (GIBCO) and antibiotics (100 IU/mL penicillin G, 100 µg/mL streptomycin, GIBCO). Cells were cultured in a humidified incubator at 37 °C in 5% CO_2.

Treatment with oligomineral water Rocchetta®

Rocchetta® mineral water was filtered using a 0.2 µm filter (ThermoFisher Scientific, Waltham, MA, USA), and stored at 4 °C. 1×10^5 HaCaT cells were plated onto 6-well culture plates and kept in culture until reached 60% confluency. Next, 10, 20, 30, 40 and 50% Rocchetta® oligomineral water diluted in medium was added to the cultures for 24, 48 and 72 h.

Analysis of cell viability

After treatments cells were washed twice with PBS 1X, incubated with trypsin/EDTA for 5 min and centrifuged at 4000 rpm for 15 min. The cell pellets were resuspended in an appropriate volume of PBS 1X, and the supernatants were collected for further experiments. Subsequently, 10 µL of each cell suspension were combined with 20 µL of Trypan blue solution. The mix was incubated for 5 min at

room temperature and the number of unstained cells (vital cells), Trypan blue stained cells (dead cell), and the total number of cells (vital and not) was determined on the hemacytometer under a microscope. The percentage of viable cells was determined dividing the number of unstained cells by the total number of cells.

UVB irradiation

HaCaT cells were cultured in complete medium alone and in 30, 40 and 50% Rocchetta® oligomineral water for 24 h. Next, medium was removed, and cells were washed twice with phosphate-buffered saline (PBS 1X, GIBCO) and irradiated using as source six Philips TL12/60W fluorescent lamps (Philips, Eindhoven, The Netherlands) emitting UVB light between 290 and 320 nm with a peak at 300 nm, for 10 min. The intensity of UVB irradiation, measured with a UV meter (Spectrolyne mod., Spectronics Corp., Westbury, NY, USA) was 60 mJ/cm^2 at 15 cm distance. After the irradiation, PBS was replaced either with complete medium or 30, 40 and 50% Rocchetta® oligomineral water and cells were let to recover for additional 24 h.

RNA extraction and real-time

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Doncaster, Australia) according to the manufacturer's instructions. The RNA yield was determined by quantifying the samples on a Nanodrop ND1000 UV-vis Spectrophotometer. cDNA was prepared using the Transcriptor High fidelity cDNA Synthesis Kit (Roche, Indianapolis, IN, USA). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR; LightCycler, Roche, Indianapolis, IN, USA) was performed to assess gene expression of *TNF*, *IL1β*, *IL6*, *COX2*, *GADD45*, *Caspase1*, *Caspase3* and *RIPK3* by *Sybr* green assay. PCR primers were designed based on published sequences, and their specificity was verified with BLAST alignment search (Table II). The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA of *18S* reference gene in the same sample. Fold induction of gene expression was calculated using the ΔΔCT method as previously described.[23]

Enzyme-Linked Immunosorbent Assay (ELISA)

TNF, *IL-1β*, and *IL-6* cytokine levels in the supernatants were assessed by ELISA using commercially available kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

TABLE II.—*Primers sequences.*

Gene	Primers sequences
18S	5′-AACCCGTTGAACCCCATT-3′
	3′- CCATCCAATCGGTAGTAGCG-5′
IL-1β	5′- TCCTGCGTGTTGAAAGATGATAA-3′
	3′- CAAATCGCTTTTCCATCTTCTTC-5′
TNF	5′-CTCTTCTGCCTGCTGCACTTTG-3′
	3′-ATGGGCTACAGGCTTGTCACTC-5′
IL-6	5′-AGACAGCCACTCACCTCTTCAG-3′
	3′-TTCTGCCAGTGCCTCTTTGCTG-5′
COX-2	5′-CGGTGAAACTCTGGCTAGACAG-3′
	3′-GCAAACCGTAGATGCTCAGGGA-5′
GADD45	5′-CGTTTTGCTGCGAGAACGAC-3′
	3′-GAACCCATTGATCCATGTAG-5′
Caspase 1	5′-AAGTCGGCAGAGATTTATCC-3′
	3′-ATGTCCGAAGCAGTGAGATT-5′
Caspase 3	5′-GTACAGAACTGGACTGTGGC-3′
	3′-ACCAGGTGCTGTGGAGTATG-5′
RIPK3	5′-TGTCTCCACGGTAAAGGATT-3′
	3′-GTTTAGCCACTCAGAAACCA-5′

Oxidative stress assayw

Oxidative stress was evaluated using the ROS-ID Total ROS/Superoxide Detection Kit (ENZO, Life Sciences, Inc., NY, USA) according to manufacturer's instructions. Briefly, 3×10^5 HaCaT cells were seeded directly onto glass slides and cultured either in complete medium or 40 and 50% Rocchetta® oligomineral water in complete medium for 24 h. Once cells reached 50-60% confluence, medium was removed, cells were washed twice with PBS and irradiated as described above. Next, PBS was removed, and cells were cultured either in complete medium or 40 and 50% Rochetta® oligomineral water for additional 24 h. Induction phase was performed addicting ROS/Superoxide Detection Solution (2X) for 30 min. ROS inducer (pyocyanin) was used as positive control. Next, ROS/Superoxide Detection Solution was removed, cells were washed twice in wash buffer, immediately overlayed with a cover slip and analyzed by means of fluorescence microscopy equipped with Fluorescein (Ex/Em: 490/525 nm) and with Rhodamine (Ex/Em: 550/620 nm) filters.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc, La Jolla, CA, USA). All data are representative of three independent experiments and expressed as mean±standard deviation. Statistical evaluation was performed by parametric One-way Anova test followed by Dunnett' multiple comparison test and statistical significance was defined as $P<0.05$.

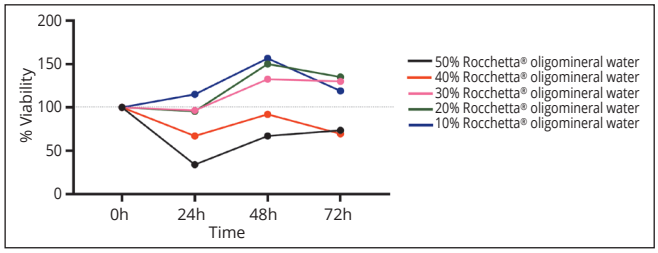

Figure 1.—Cell viability was assessed at 24, 48 and 72 h after treatment with increasing percentage of Rocchetta® mineral water (from 10 to 50%). Cellular viability rate and statistical significance were determined compared to the 100% viability of untreated control cells. Data are expressed as mean±SD of three independent experiments, each performed in triplicate. Statistical two-way Anova was performed.

Results

Effects of oligomineral water on cell viability

To establish the effect of Rocchetta® oligomineral water on cell viability, HaCaT cell were cultured with increasing concentrations of water (from 10 to 50%) for up to 72 h (Figure 1). The number of viable HaCaT cell did not change when incubated with 10, 20 and 30% Rocchetta® oligomineral water for 24 and 72 h while increased at 48 h. On the other hand, 40 and 50% Rocchetta® oligomineral water decreased HaCaT viability at 24 h (by 65% and 35% respectively) and 72 h (by 69.5% and 73.5% respectively). To verify the total absence of inflammatory stimulus due to the water and to choose the optimal dilution and timing, gene expression of *TNF*, *IL-1β*, *IL-6*, *COX-2* and Caspase-1 was assessed. At 48 h no difference was observed compared to control. Hence, 48 h was chosen as the experimental timing, and 30, 40 and 50% as dilutions for the subsequent experiments.

Protective effects of oligomineral water after UVB irradiation on main mediators of inflammation

To verify the protective effects of Rocchetta® oligomineral water on UVB-irradiated cells, mRNA expression of *TNF*, *IL1β*, *IL6*, *COX2* and *Caspase1* was assessed in HaCaT cell pretreated with 30, 40 and 50% Rocchetta® oligomineral water for 24 h, subsequently irradiated with UVB 60 mJ/cm² for 10 min and let to recover for further 24 h (Figure 2A). mRNA levels of all analyzed genes were significantly (P<0.05) reduced by

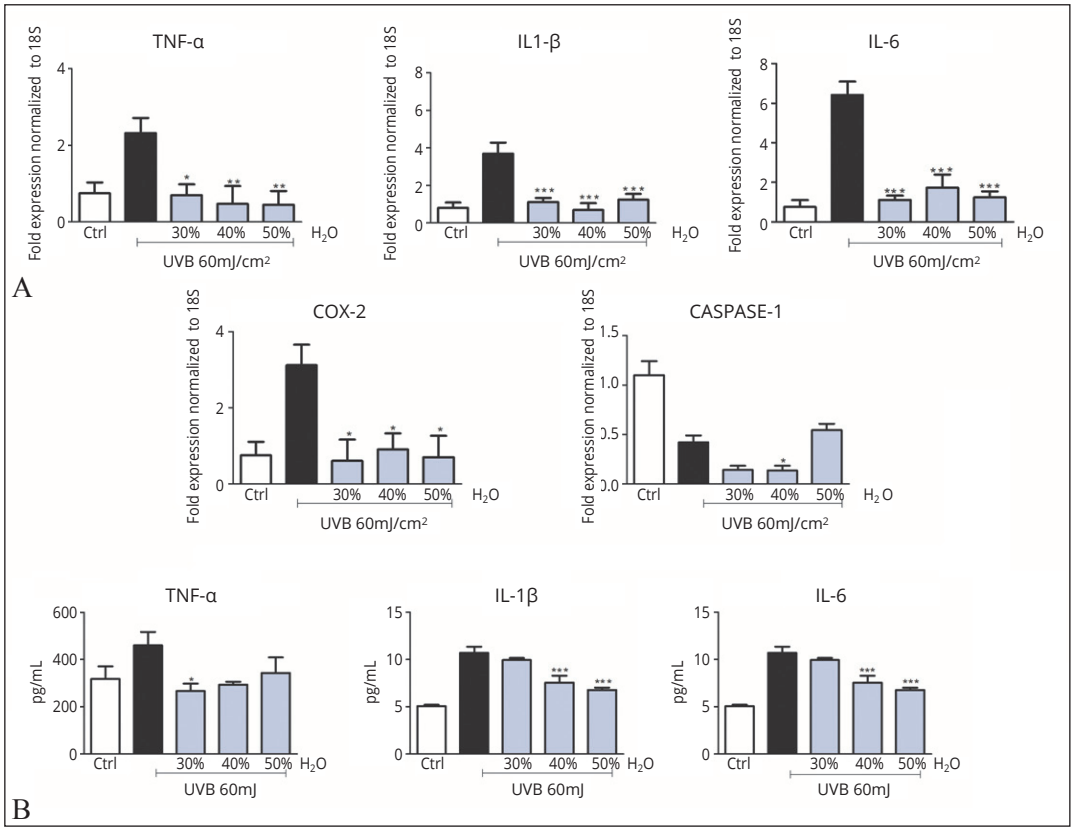

Figure 2.—A) *TNF*, *IL1β*, *IL6*, *COX2*, and *CASPASE1* gene expression was assessed in HaCaT cell pretreated with 30, 40 and 50% Rocchetta® oligomineral water for 24 h, subsequently irradiated with UVB 60 mJ/cm² for 10 min and let to recover for further 24 h. Untreated Hacat cells and UVB-irradiated cells were used as internal control. Values are normalized to 18 S and expressed as mean±standard deviation. (B) *TNF*, *IL-1β* and *IL-6* protein levels were measured in HaCaT cell pretreated with 30, 40 and 50% Rocchetta® oligomineral water for 24 h, subsequently irradiated with UVB 60 mJ/cm² for 10 min and let to recover for further 24 h. Statistical significance was assessed using parametric One-way Anova test followed by Dunnett' multiple comparison test and statistical significance was defined as *P<0.05; ***P<0.001.

Rocchetta® oligomineral water at all tested concentration. Thus, exposure to Rocchetta® oligomineral water for 48 h reduced UV-induced inflammatory mediators in keratinocytes. In keeping with the gene expression data, 40 and 50% Rocchetta® oligomineral water significantly reduced the level of secreted *IL-1β* and *IL-6* in the culture. *TNF* protein was significantly reduced by 30% Rocchetta® oligomineral water with a downward trend at 40 and 50% (Figure 2B).

Protective effects of oligomineral water on UVB-induced cell death

To investigate the protective effect of Rocchetta® oligomineral water on UVB-induced cell death, gene expression of *GADD45*, *Caspase3* and *RIPK3*, key mediators of ell DNA damage, programmed cell death (apoptosis) and necrosis, was also investigated. Albeit not statistically significant, we detected a downward trend in the expression of these markers in cells pretreated with Rocchetta® oligomineral water prior to UVB irradiation as described above (Figure 3).

Protective effects of oligomineral water on ROS/superoxide production UVB-induced

Finally, we evaluated the antioxidant activity of Rocchetta® oligomineral water by measuring total ROS/RNS and superoxide production as markers of oxidative stress after UVB irradiation. Pretreatment with 40 and 50% Rocchetta® oligomineral water reduced the amount of total ROS/RNS produced compared to control UVB irradiated cells (Figure 4). The amount of total ROS/RNS showed a stronger downward trend by 40% as compared to 50% Rocchetta® oligomineral water.

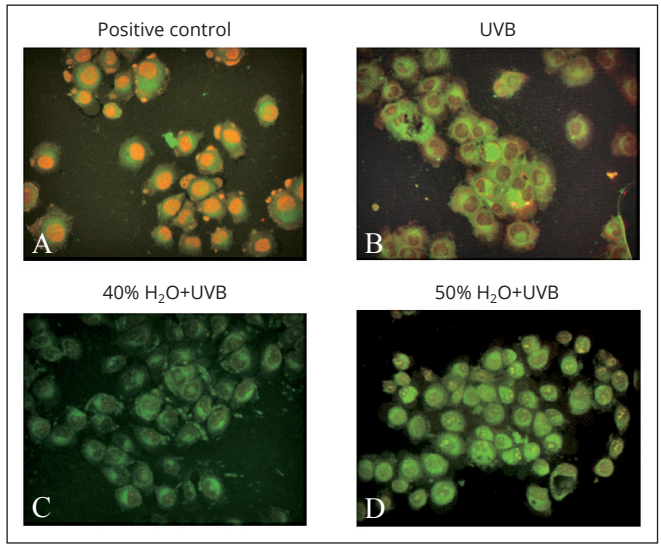

Figure 4.—Immunofluorescence staining of total ROS/RNs (green) and superoxide (orange) was performed in HaCaT cells after 24 h of irradiation with UVB (60 mJ/cm²) in cells cultures pretreated for 24 h with oligomineral water (40 and 50%). Positive control (A); UVB (60 mJ/cm²) (B); UVB (60 mJ/cm²) and 40% of oligomineral water (C); UVB (60 mJ/cm²) and 50% of oligomineral water (D). The amount of total ROS/RNS showed a stronger downward trend by 40% as compared to 50% Rocchetta® oligomineral water. Magnification 20×.

Discussion

In this study, we evaluated the anti-inflammatory, antioxidant and antiapoptotic effects of Rocchetta® oligomineral water in UVB-irradiated immortalized human keratinocytes. We have shown that Rocchetta® oligomineral water is well tolerated by the cells and displays anti-inflammatory, antioxidant and antiapoptotic proprieties when used prior keratinocyte UVB irradiation. The skin serves as

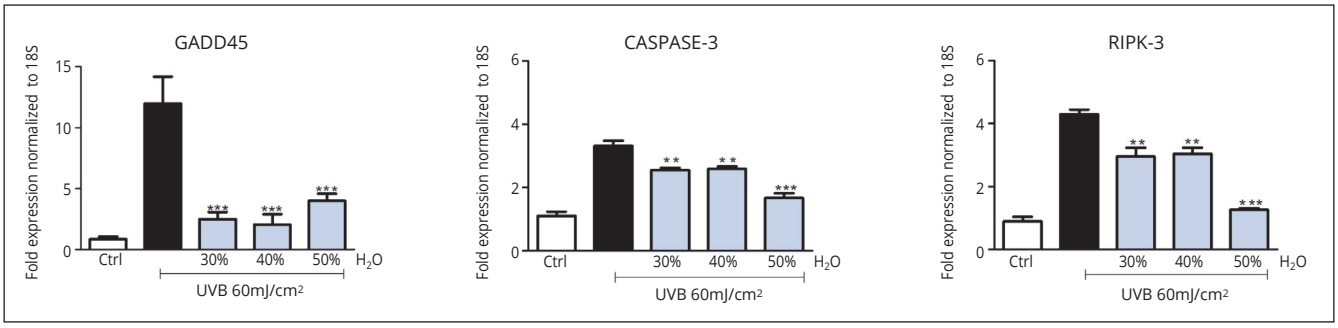

Figure 3.—*GADD45*, *CASPASE3* and *RIPK3* gene expression was assessed after 24 h of irradiation with UVB (60 mJ/cm²) in cells pretreated for 24 h with oligomineral water (30, 40 and 50%). Gene expression after oligomineral water incubation and UVB irradiation was compared to the values of irradiated cells alone. Untreated Hacat cells and UVB-irradiated cells were used as internal control. Values are normalized to the housekeeping gene *18S* and expressed as mean±standard deviation. Statistical significance was assessed using parametric One-way Anova test followed by Dunnett' multiple comparison test.
Statistically significant, P<0.01; * statistically significant, P<0.001.

key protective barrier against injuries, xenobiotics, and microbial pathogens and plays a key role in the maintenance of homeostatic and thermostatic balance via several mechanisms including prevention of water loss.[6, 7] Several reports have previously suggested that oligomineral water may have a beneficial immunomodulatory role in skin physiology. Keratinocytes are the major target of UV, and they play a key role in a first line of body defenses. In this study, HaCaT keratinocytes cell line was cultured with increasing concentrations of Rocchetta® oligomineral water (from 10% to 50%) for up to 72 h to establish the effect of the water on cell viability. Results showed that at 48 h 10%, 20%, 30% and 40% Rocchetta® oligomineral water either increased (10%, 20%, 30%) or did not affect the cell number viability (40%). Our findings are in accordance with a study published by Nicoletti *et al*. on primary fibroblast cultured in presence of different concentration of spring water at different time points for up 7 days. The authors identified 48 h as well as 10%, 20%, 30% and 40% spring water as the best time window and water concentration for cell viability. Among the external stimuli that affect the skin, ultraviolet (UV) radiation, which is frequently encountered in everyday life, is a major environmental factor of skin damage. It is known that most skin damage caused by UV rays occurs with UVB (280-320 nm), and the wavelength of the UVB region is absorbed in the epidermal layer of the skin.[24] Accumulating evidence suggests that UVB irradiation not only induces nuclear DNA damage but also causes membrane destruction, resulting in cell loss or apoptosis.[25, 26] It has been reported that oxidative stress on reactive oxygen species (ROS) produced by UVB irradiation is a cause of skin inflammation.[26] Therefore, we have evaluated the anti-inflammatory effects of Rocchetta® oligomineral water on the modulation of inflammatory molecules such as *TNF*, *IL1β*, *IL6*, *COX2* and *Caspase1* in UVB induced keratinocytes which were reduced at both mRNA and protein level. The reduced expression of *Caspase1* suggest a possible modulatory effect of Rocchetta® oligomineral water also on the inflammasome complex, which, to the best of our knowledge, has not been reported in the literature before. Moreover, gene expression results were supported by a reduced level of IL-1 β and *IL-6* production in 30, 40% Rocchetta® oligomineral water cultured HaCaT cells for 48 h. Our expression results are in line with published studies reporting an anti-inflammatory effect of thermal water in HaCaT cells and antigen presenting cell. Decreased expression of *TNF*, *IL1α* and *VEGF* in cells culture with thermal waters was also reported.[2] Prolonged exposure to UVB results in exacerbated innate and adaptative immune response as well as DNA damage leading the cells to an early apoptosis/necrosis.[27] Therefore, we investigated a possible protective effect of Rocchetta® oligomineral water on UVB -induced cell death by looking at the expression of key mediators of DNA cell damage, programmed cell death apoptosis and necrosis such as *GADD45*, *Caspase3* and *RIPK3*. Our data show a reduction in the expression of the investigated genes. Finally, we evaluated the antioxidant activity of Rocchetta® oligomineral water in UVB-irradiated keratinocytes by measuring the total ROS/RNS and superoxide production. Our data highlighted a role of Rocchetta® mineral water in protecting from the formation reactive oxygen species. These results were in line with the data by Zöller *et al*. who observed a reduction in formation of reactive oxygene species in UVB irradiated HaCaT cells cultured with thermal spring waters and natural mineral drinking waters. Taken together, our results show that Rocchetta® oligomineral water may contribute to the reduction of parameters associated with inflammation in keratinocytes.

Limitations of the study

We acknowledge the presence of some limitations in the study, such as the analysis of a limited number of key inflammatory cytokines and apoptotic/necrosis mediator based upon literature.

Conclusions

In conclusion, our results highlight a possible protective role of Rocchetta® oligomineral water in modulating the cutaneous inflammatory response to external triggers and injuries. Further studies will be required, using different cell types or real human skin to validate these results and to explore more in depth the beneficial properties of Rocchetta® oligomineral, identify the molecular and cellular mechanisms underpinning Rocchetta® oligomineral water anti-inflammatory, antiapoptotic and antioxidant proprieties.

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Conflicts of interest.—Gabriella Fabbrocini has received a research grant from CoGeDi International SpA. The other authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Funding.—This study was partially funded by CoGeDi Internation SpA.

Authors' contributions.—Giuseppina Caiazzo and Melania Parisi equally contributed to the manuscript and share first-authorship. Giuseppina Caiazzo, Melania Parisi, and Gabriella Fabbrocini have given substantial contributions to the study conception; Roberta Di Caprio and Lucia Gallo contributed to the study design and methodology; Maria Quaranta contirbuted to the software management; Sara Cacciapuoti, Maria A. Luciano and Maria Quaranta contributed to the study validation; Giuseppina Caiazzo and Melania Parisi contributed to the formal analysis; Giuseppina Caiazzo contributed to the study investigation; Melania Parisi and Lucia Gallo contributed to the data collection; Maria Quaranta and Roberta Di Caprio contributed to the data interpretation; Giuseppina Caiazzo and Melania Parisi contributed to the original draft preparation; Maria Quaranta and Gabriella Fabbrocini contributed to the manuscript draft, editing and critical revision; Sara Cacciapuoti contributed to the study visualization; Gabriella Fabbrocini contributed to the study supervision and to the project administration; Maria A. Luciano contributed to the funding acquisition. All authors read and approved the final version of the manuscript.

History.—Article first published online: March 21, 2022. - Manuscript accepted: January 24, 2022. - Manuscript revised: January 21, 2022. - Manuscript received: November 12, 2021.