




Protective role of Portuguese natural mineral waters on skin aging: in vitro evaluation of anti-senescence and anti-oxidant properties

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Abstract

Natural mineral waters (NMWs) emerge from the earth as springs and their beneficial therapeutic effect has been empirically recognized in different countries. Portugal has diverse NMW resources that are sought for the relief of different afflictions including dermatological complications. However, there is a lack of scientific validation supporting this empiric knowledge. In this study, we aimed to screen the in vitro bioactivity of Portuguese NMWs with different chemical profiles, namely sulfurous/bicarbonate/sodic (SBS), bicarbonate/magnesium, sulfated/calcic, sulfurous/chlorinated/sodic, sulfurous/bicarbonate/fluoridated/sodic, and chlorinated/sodic, focusing on aging-related skin alterations. Mouse skin fibroblasts and macrophages were exposed to culture medium prepared in different NMWs. Cellular viability was evaluated by MTT assay and etoposide-induced senescence was analyzed through the beta-galactosidase staining kit. Wound healing was investigated by the scratch assay, and phototoxicity/photoprotection after UVA irradiation was evaluated using a neutral red solution. ROS production was quantified using the 2',7'-dichlorofluorescein diacetate dye, and the activity of superoxide dismutase (SOD) was analyzed by a commercial kit after lipopolysaccharide exposure. NMWs within the SBS profile demonstrated anti-senescence activity in skin fibroblasts, along with a variable effect on cellular viability. Among the tested NMWs, two decreased cellular senescence and preserved cell viability and were therefore selected for subsequent studies, together with a SBS NMW with therapeutic indications for dermatologic diseases. Overall, the selected NMW promoted wound healing in skin fibroblasts and activated SOD in macrophages, thus suggesting an anti-oxidant effect. None of the NMWs prevented phototoxicity after UV irradiation. Our results shed a light on the anti-aging potential of Portuguese NMW, supporting their putative application in cosmetic or medical products.

Keywords Spring waters · Wound healing · Anti-aging · Cell viability · Oxidative stress

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Introduction

Natural mineral waters (NMWs) are defined as natural aqueous solutions formed under specific geological conditions and have three fundamental characteristics: originate naturally from the earth as “springs,” being pure, and having some therapeutic potential or health beneficial effect (Ferreira et al. 2010). The therapeutic properties of NMWs were widely recognized long before the development of modern medical treatments, and nowadays, balneotherapy, which uses medical mineral waters, medical peloids, and/or natural gasses for therapeutic purposes, is considered an useful alternative for several conditions in many Health Resort Medicine Centers all over the globe (Hercogova et al. 2002; Gomes et al. 2013; Fillimonova et al. 2022).

Portuguese NMWs have been increasingly requested for the treatment of numerous afflictions of neuromuscular, gastrointestinal, articular, respiratory, and epidermic/dermic nature (Rebello et al. 2015). Actually, in Portuguese territory, there are about 50 thermal centers mainly in the Northern and Central regions of the country (Coutinho et al. 2015; Sozo et al. 2021). Nevertheless, less than 50% have recognized dermatological therapeutic indications. Specifically, in the central region, only four thermal centers have recognized therapeutic indications for skin conditions (Oliveira et al. 2019).

NMWs with different chemical compositions are sought for different dermatologic afflictions. Concerning Portuguese NMWs, chlorinated waters are specifically requested for healing non-exudative afflictions, sulfurous waters for seborrhea and acne, chronic eczemas and psoriasis, and hyposaline waters, with a high content in silica, are also indicated for gynecological conditions along with diverse dermatological diseases (Araujo et al. 2017).

The major dermatological conditions that are frequently treated by balneotherapy, with a high rate of success, are psoriasis and atopic dermatitis (Matz et al. 2003). Other conditions include acne vulgaris, alopecia areata, contact dermatitis, dyshidrotic dermatitis, eczema, mycosis fungoides, necrobiosis lipoidica, palmoplantar keratosis, parapsoriasis group, pityriasis rubra pilaris, psoriasis, rosacea, scleroderma, sebopsoriasis, seborrheic dermatitis, ulcer (chronic), urticaria pigmentosa and vitiligo (Matz et al. 2003). Despite this long list of skin ailments that benefit from exposure to NMW, the efficacy of balneotherapy and the mechanisms underlying the bioactive effects of NMW are only partially understood and presumably include chemical, thermal, mechanical, and immunomodulatory effects (Lee et al. 2012).

It is known that water plays an important role in the dermatological treatment, through skin hydration and hygiene, and as a vehicle for different substances (Stettler et al. 2021). Moreover, water comprises about 60% of the skin weight, and the control of the exchange of water between the stratum corneum and the environment is an important function of the skin and an indicator of the integrity of the stratum corneum barrier

(Nunes and Tamura 2012). Furthermore, skin homeostasis is achieved through a constant crosstalk between its major components: dermal fibroblasts, epidermal keratinocytes, nerves, intradermal adipocytes, and immune cells, such as macrophages (Thulabandu et al. 2018). The progressive decline of skin cell function and loss of homeostasis, along with other factors such as inflammaging, leads to the skin aging process (Ganceviciene et al. 2012; Maru et al. 2014; Pilkington et al. 2021). Cells that lose their functionality are called senescent cells and skin cells as fibroblasts, keratinocytes and melanocytes in their senescence state have beta-galactosidase activity and may secrete several pro-tumorigenic and pro-inflammatory cytokines, as IL-1, IL-6, IL-8, HGMB1, metalloproteinases, and p21, p16, and p53 cell cycle regulators that contribute to aging and many age-related pathologies. Cellular senescence is presently recognized as a hallmark of aging (Wang and Dreesen 2018; Lämmermann et al. 2018).

Although the regulatory function of chemical elements present in NMWs on aging-related events such as cell proliferation and renewal, as well as on oxidative stress and inflammation, has already been identified (Table 1), the lack of scientific support drives several thermal centers to evaluate the influence of their NMWs (i.e., Uriage, Avène, and La Roche-Posay) on skin health. These particular NMW have already demonstrated anti-oxidant activity (Joly et al. 2000; Merial-Kieny et al. 2011). Also, other NMWs have been positively correlated with anti-oxidant and anti-inflammatory properties, skin regeneration, and cellular proliferation, in both in vitro and in vivo studies (Hercogova et al. 2002; J. Richard et al. 2010; Bieber 2011; Faga et al. 2012; Nicoletti et al. 2016; Silva et al. 2020a).

As for Portuguese NMW, some efforts have been made in order to assess and promote their clinical benefits. São Pedro do Sul NMW has been shown to enhance skin barrier recovery after irritation induced by sodium lauryl sulfate in healthy human volunteers (Ferreira et al. 2010). However, the mechanisms underlying the observed anti-irritant effects were not evaluated in vitro and its role on skin aging was not determined. Also, in previous studies, our own team have demonstrated the antimicrobial, anti-inflammatory and anti-proliferative effects of Portuguese NMWs (Oliveira et al. 2019, 2020; Silva et al. 2020a). Some topical products with Portuguese NMW from Cró and Monfortinho thermal centers have also been developed and evaluated (Almeida et al. 2019; Nunes et al. 2019). Still, their effect in skin aging remains unstudied.

Therefore, with this study, we aimed to characterize the anti-senescence and anti-oxidant properties of individual NMW from the central region of Portugal, as markers of their anti-aging potential. For that purpose, we analyzed the viability and induced senescence of representative skin cells after NMW treatment, and for the most promising NMWs, migration, phototoxicity, and key oxidative stress parameters as ROS production and activity of the anti-oxidant enzyme superoxide dismutase (SOD) were further evaluated.

Table 1 Physiological function and/or therapeutic application of chemical elements present in natural mineral waters. Adapted from Nunes and Tamura (2012) and Coutinho et al. (2015)

Chemical element	Physiological function	Therapeutic application
Aluminum		Acute dermatitis
Arsenic		Psoriasis
Boron	Cell renewal	Wound healing
Copper	Antioxidant Skin barrier recovery	
Magnesium	Cell renewal	Acute dermatitis
Sulfur	Cell regeneration Keratoplastic or keratolytic (dose-dependent) Antioxidant	Antibacterial and antifungal
Silica	Synthesis of collagen and elastin Cell metabolism Emollient effect	Psoriasis
Sodium	Cell renewal	
Calcium	Cell division Cell differentiation Cell membrane permeability Cell proliferation Anti-inflammatory	
Potassium	Synthesis of nucleic acids and proteins Cell metabolism	
Manganese	Cell renewal Wound healing Skin barrier	
Selenium	Antioxidant and protection against UVB radiation	Tinea versicolor Seborrheic dermatitis
Zinc	Antioxidant Cell renewal Modulation of inflammation	

Methods

Thermal center recruitment

Thirteen thermal centers belonging to the central region of Portugal were enrolled in this study. NMW were collected from the spring/borehole of each thermal center, after purging and according to the appropriate procedures (APHA 2005). NMW samples were collected in several sterile flasks, sealed and transported in refrigerated thermal boxes with frozen accumulators, and then stored at 4 °C in the dark until used. Each flask was open once, and for each experiment, a new one was opened. At reception, samples were analyzed for their organoleptic features (odor, color, deposit, and aspect) and a microbiological quality control was performed for each sample as described in *Farmacopeia Portuguesa* 9 to ensure water sterility (INFARMED—Instituto Nacional da Farmácia e do Medicamento 2009). NMWs that were not sterile at reception were filtered using a 0.2 µm pore filter (VWR, Alfragide, Portugal). Each thermal center provided the physicochemical composition of their NMW (as disclosed in Supplementary Table S1 and in Oliveira et al. 2019).

NMWs were divided by their ionic profile and using the classification adopted by “Termas de Portugal” and

Portuguese Directorate General for Energy and Geology (DGEG). Table 2 describes the NMW codification and division by their ionic profile and addresses their regulated use. NMWs were divided as follows: sulfurous/bicarbonate/sodic (SBS) — 8 water samples; sulfurous/bicarbonate/fluoridated/sodic (SBFS) — 1 water sample; chlorinated/sodic (CS) — 1 water sample; bicarbonate/magnesium (BM) — 1 water sample; sulfated/calcic (SC) — 1 water sample; and sulfurous/chlorinated/sodic (SCS) — 1 water sample.

To ensure the confidentiality of the thermal centers involved, each center was uniquely labeled.

Preliminary tests of the effect of NMWs on murine skin fibroblast viability and etoposide-induced cell senescence

Cell lines

Murine skin fibroblast cell line NIH/3T3 (ATCC Cat# CRL-1658, RRID:CVCL_0594) was grown in DMEM medium (LTID 31,600–083, Life Technologies, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Life Technologies), 25 mM glucose (Sigma-Aldrich, St. Louis, MO, USA), 35.9 mM sodium bicarbonate

Table 2 Classification and therapeutic application (grey cells) of the Portuguese natural mineral waters enrolled in the study (adapted from Direção-Geral da Saúde 1989 and Termas de Portugal — <https://termasdeportugal.pt/rede-termas>)

Classification	Mineralization	Ionic Profile	NMW Code n°	Therapeutic applications					
				Endocrine metabolism	Circulatory system	Urinary System	Digestive System	Respiratory System	Skin
Poorly mineralized	Sulphurous/ Bicarbonate/ Sodic (SBS)	1							
		2							
		3							
		4							
		5							
		6							
		12							
Hyposaline	Sulphurous/ Bicarbonate/ Fluoridated/ Sodic (SBFS)	15							
		16							
		9							
Hypersaline	Sulphated/ Calcic (SC)	10							
		11							
	Sulphurous/ Chlorinated/ Sodic (SCS)								

Grey cells represent the therapeutic applications of each NMW

(Sigma-Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich). Cells were maintained at 37 °C and 5% CO₂ humidified atmosphere.

To determine the effects of each NMW on the cell line model, culture medium (DMEM LTID 31,600–083 supplemented with 10% FBS, 25 mM glucose, 35.9 mM sodium bicarbonate, 100 U/mL penicillin, and 100 µg/mL streptomycin) was completely prepared in NMW (without dilution) instead of Milli-Q-type water that was used to prepare the control culture medium, further referred as control (CTRL). NMW-containing medium was always prepared from a newly opened flask every time that was needed.

Cell viability

Cell viability of skin fibroblasts treated with Portuguese NMW was determined by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described elsewhere (Oliveira et al. 2020).

Briefly, 1×10^4 cells/well (200 µl) were seeded onto 96-well plates in DMEM medium supplemented with FBS, 25 mM glucose, 35.9 mM sodium bicarbonate, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO₂ humidified atmosphere. After 24 h, the cells were exposed to 200 µl of medium prepared with different NMWs for an additional 24 h period. Twenty microliters of MTT solution (5 mg/mL) was added to each well and the plates were further incubated at 37 °C and 5% CO₂ for 4 h. Finally, the dark blue formazan crystals were dissolved using dimethyl sulfoxide (Sigma-Aldrich) and the quantification was performed using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, USA) at 590 nm, with a reference wavelength of 620 nm. Results were expressed as percentage of control.

Etoposide-induced senescence

Senescence was evaluated using a beta-galactosidase staining kit according to the manufacturer's instructions (Cell Signaling Technology, MA, USA), as previously described (Oliveira et al. 2020). 3T3 cells (2.5×10^4 per well) were seeded in 12-well plates in 1 mL DMEM medium supplemented with FBS, 25 mM glucose, 35.9 mM sodium bicarbonate, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO₂ humidified atmosphere and, after 24 h, senescence was induced by the administration of 12.5 µM of etoposide. After 24 h, etoposide was removed; cells were washed with PBS 1× and then incubated with 1 mL of medium prepared with different NMWs or Milli-Q water (control), as described above. Morphology was evaluated daily by microscopic visual inspection. After 72 h, 1× fixative solution provided by the commercial kit was added and maintained at room temperature for 15 min. Several PBS washes were performed and the cells were incubated overnight with beta-galactosidase staining solution at 37 °C. Different fields were observed considering the development of blue color and eight images for condition were acquired. A distinct blue color staining was indicative of beta-galactosidase activity. Quantitative analysis was performed using ImageJ, and the percentage of senescent cells to total cells was calculated (mean ± SEM).

Evaluation of NMW effect on cell migration and phototoxicity of murine skin fibroblasts and on anti-oxidant activity of murine macrophages

Cell lines

Murine skin fibroblast cell line NIH/3T3 (ATCC Cat# CRL-1658, RRID:CVCL_0594) was grown as described in the “Methods” section.

RAW 264.7, a mouse leukemic monocyte macrophage cell line (ATCC Cat# TIB-71, RRID:CVCL 0493), was cultured in DMEM medium (LTID 31,600–083, Life Technologies) pH 7.4, supplemented with 10% non-inactivated FBS (Life Technologies), 25 mM glucose (Sigma-Aldrich), 17.95 mM sodium bicarbonate (Sigma-Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich). Cells were maintained at 37 °C and 5% CO₂ humidified atmosphere.

The medium with NMW for cell stimuli was prepared and added as described in the “Methods” section.

Migration assay

The migration of 3T3 cells was investigated by a scratch-wound assay, as previously described (Ferreira et al. 2012). Prior the assay, using a scalpel, two parallel lines were carved on the underside of each well. These lines served as a guidance axis together with the line provided by the scratch wound. Then, 3T3 cells were seeded at a density of 2.5×10^5 cells per well, in 12-well plates in 1 mL DMEM medium supplemented with FBS, 25 mM glucose, 35.9 mM sodium bicarbonate, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO₂ humidified atmosphere. At 95% confluence, a perpendicular scratch was performed using a P10 pipette tip. Photographs were immediately taken ($T=0$ h) using an inverted microscope (Olympus CKX41, objective of 10×) and an Olympus digital camera (DC 6 V). Cells were then exposed to NMW mediums or Milli-Q-type water (control), using 2% FBS (v/v) in order to diminish cellular proliferation, and new photographs were taken in the same area after 12 h ($T=12$ h). The number of cells that migrate to the cell-free region (wound) was then quantified using ImageJ software.

Phototoxicity

Phototoxicity assay was performed accordingly with the OECD 432 — In Vitro 3T3 NRU Phototoxicity Test (2019). Briefly, 1×10^4 3T3 cells/well were seeded in two 96-well plates (one for irradiation and the other for control without irradiation) in DMEM medium (LTID 31,600–083), supplemented with 10% inactivated FBS, 25 mM glucose, 17.95 mM sodium bicarbonate, 100 U/mL penicillin, and 100 µg/mL streptomycin, and kept at 37 °C and 5% CO₂ during 24 h. Afterward, cells were incubated for 1 h at 37 °C and 5% CO₂ with medium without phenol red (D2902, Sigma-Aldrich, with 1% pen-strep and 10% FBS) prepared with NMW or Milli-Q-type water (control). The plate was then irradiated at a distance of 35.5 cm with the Ultra-Vitalux 300 W lamp (OSRAM) ensuring a 1.7mW/cm² of UVA radiation during 10 min, and the non-irradiated plate was maintained at the same temperature in the dark. Cells were then washed twice with DPBS (Sigma D8662) and covered with DMEM without phenol red (with 10% FBS and 1%

pen-strep) during 18 h at 37 °C and 5% CO₂. The medium was removed and neutral red solution (50 µg/mL in culture medium) was added. After 3 h of incubation at 37 °C and 5% CO₂, cells were washed twice with DPBS and a solution containing 50% ethanol absolute and 1% acetic acid in distilled water was added to extract the dye. After 10 min on a plate shaker, the absorbance of neutral red was measured at a wavelength of 540 nm in the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). Chlorpromazine was used as positive control (0, 0.1, 0.5, 1, 1.5, 2, 5, 10, and 20 µg/ml for the irradiated plate and 0, 0.5, 1, 5, 10, 20, 30, 40, and 50 µg/ml for the non-irradiated plate) to guarantee the quality of the assay.

Reactive oxygen species (ROS) production

The production of intracellular reactive oxygen species (ROS) was quantified on murine macrophages through the 2',7'-dichlorofluorescein diacetate (H₂DCFDA, Sigma), a cell-permeable non-fluorescent probe that is rapidly oxidized to the fluorescent 2',7'-dichlorofluorescein, in the presence of intracellular ROS.

Raw 264.7 cells were plated at a density of 2×10^4 cells/well in 96-well black plates with clear bottom in DMEM medium (LTID 31,600–083), supplemented with 10% non-inactivated FBS, 25 mM glucose, 17.95 mM sodium bicarbonate, 100 U/mL penicillin, and 100 µg/mL streptomycin, and kept at 37 °C and 5% CO₂ during 24 h, and then treated with the same medium prepared with NMW or control simultaneously with 1 µg/mL lipopolysaccharide (LPS) for 18 h. After treatments, the cell medium was removed and replaced with Hanks' balanced salt solution (HBSS) containing 5 µM H₂DCFDA and 0.5 g/mL Hoechst solution for 30 min at 37 °C in the dark and in a humidified atmosphere with 5% CO₂. The dye solution was replaced by HBSS and the fluorescence was measured in a SpectraMax Gemini EM Microplate Reader (Molecular Devices) at excitation and emission wavelengths of 485/530 nm and 350/461 nm, respectively. The results are expressed as percentage of control of the ratio H₂DCFDA/Hoechst absorbance in each condition.

Superoxide dismutase (SOD) activity

In order to evaluate the SOD activity, one of the most important anti-oxidant enzymes, 7×10^7 Raw 264.7 cells were seeded in 6-well plates with DMEM medium (LTID 31,600–083), supplemented with 10% non-inactivated FBS, 25 mM glucose, 17.95 mM sodium bicarbonate, 100 U/mL penicillin, and 100 µg/mL streptomycin, and kept at 37 °C and 5% CO₂ during 24 h, and then treated with the same medium prepared with NMW or control simultaneously with 1 µg/mL of LPS for 24 h and incubated at 37 °C in a 5% CO₂ atmosphere. Cells were collected, total protein was extracted using RIPA Buffer (150 mM NaCl; 50 mM Tris-HCl, pH 8.0; 1%

Nonidet P-40; 0.5% v/v sodium deoxycholate; 0.1% v/v SDS; 2 mM EDTA), and SOD activity was measured using the SOD Assay Kit-WST (Sigma-Aldrich) following manufacturer's instructions. Briefly, this kit uses the WST-1 substrate (tetrazolium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with superoxide anion, occurring at 37 °C, is linearly related with the activity of xanthine oxidase and inhibited by SOD. The generation of formazan was monitored by measuring the absorbance at 450 nm in a xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad) and SOD activity was quantified as the percentage of reaction inhibition rate.

Statistical analysis

All assays were executed in triplicate in at least three independent experiences, and all values were expressed as percentages normalized to the control (medium prepared in Milli-Q-type water).

The results are presented as mean ± SEM and were analyzed with *t*-test or one-way ANOVA with Tukey's multiple comparison test, using GraphPad Prism (GraphPad Software, California, USA). $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significant.

Results

The following sections are divided to present first the preliminary results obtained for all NMWs under study and, secondly, results from further investigations that were only performed with three selected NMWs. Table 3 presents

an overview of the obtained results. Details on specific results for each bioactivity are addressed in the subsections below.

Preliminary results of the evaluation of NMWs on murine skin fibroblast viability and etoposide-induced cell senescence

NMWs affect the viability and senescence of skin fibroblasts

The effect of the NMW on viability of fibroblasts was evaluated and the results are presented in Fig. 1.

The results achieved demonstrated that the NMWs 1 and 3, both belonging to the SBS ionic profile, increased the cell viability of fibroblasts, while it was decreased by NMWs 4 and 5 belonging to the same ionic profile (Fig. 1A). No significant changes in cell viability were noted in the fibroblasts treated with the other SBS NMWs (Fig. 1A). In what concerns cell viability of fibroblasts exposed to the BM, SCS, SBFS, and CS NMW, no significant alterations were observed (Fig. 1B–E). On the other hand, a decrease in cell viability was found in fibroblasts treated with NMW 16 belonging to the CS ionic profile (Fig. 1F).

NMWs have a positive impact on skin fibroblast senescence

The effect of NMWs, belonging to different ionic profiles, on preventing fibroblast senescence triggered by etoposide is presented in Fig. 2.

The results demonstrate that all NMWs with a SBS profile (1, 2, 3, 4, 5, 6, 12, and 13) significantly reduce the number of etoposide-induced senescent cells when

Table 3 Schematic representation of the results obtained for skin fibroblasts and macrophages, after natural mineral water (NMW) exposure

NMW	NMW activity						
	Fibroblasts				Macrophages		
	Viability	Senescence	Migration	Phototoxicity	ROS production	SOD activity	
SBS	1	↑	↓	↓	–	–	↑
	2	–	↓	↑	–	–	↑
	3	↑	↓	↑	–	–	↑
	4	↓	↓				
	5	↓	↓				
	6	–	↓				
	12	–	↓				
	13	–	↓				
SBFS	15	–	↓				
CS	16	↓	↑				
BM	9	–	–				
SC	10	–	↓				
SCS	11	–	↓				

↓ decreased activity; ↑ increased activity; – no effect

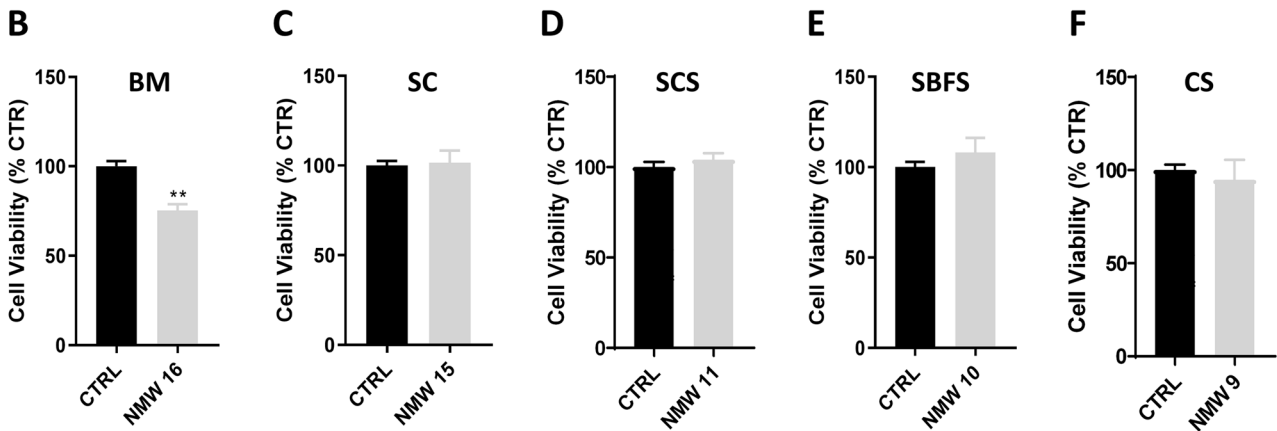
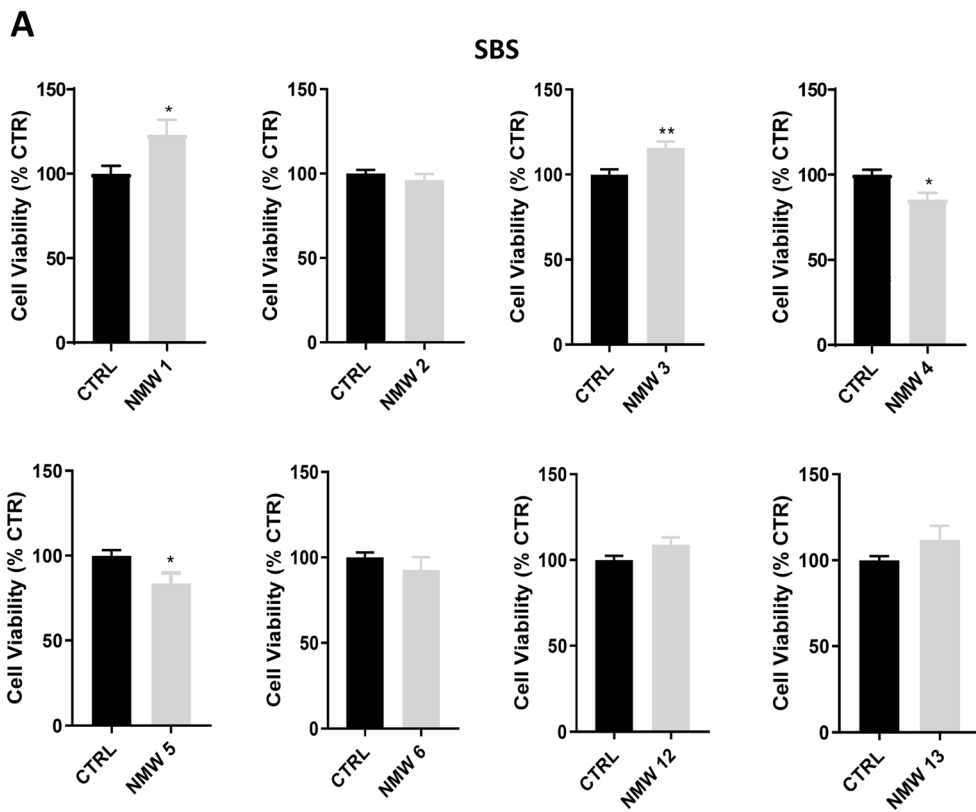


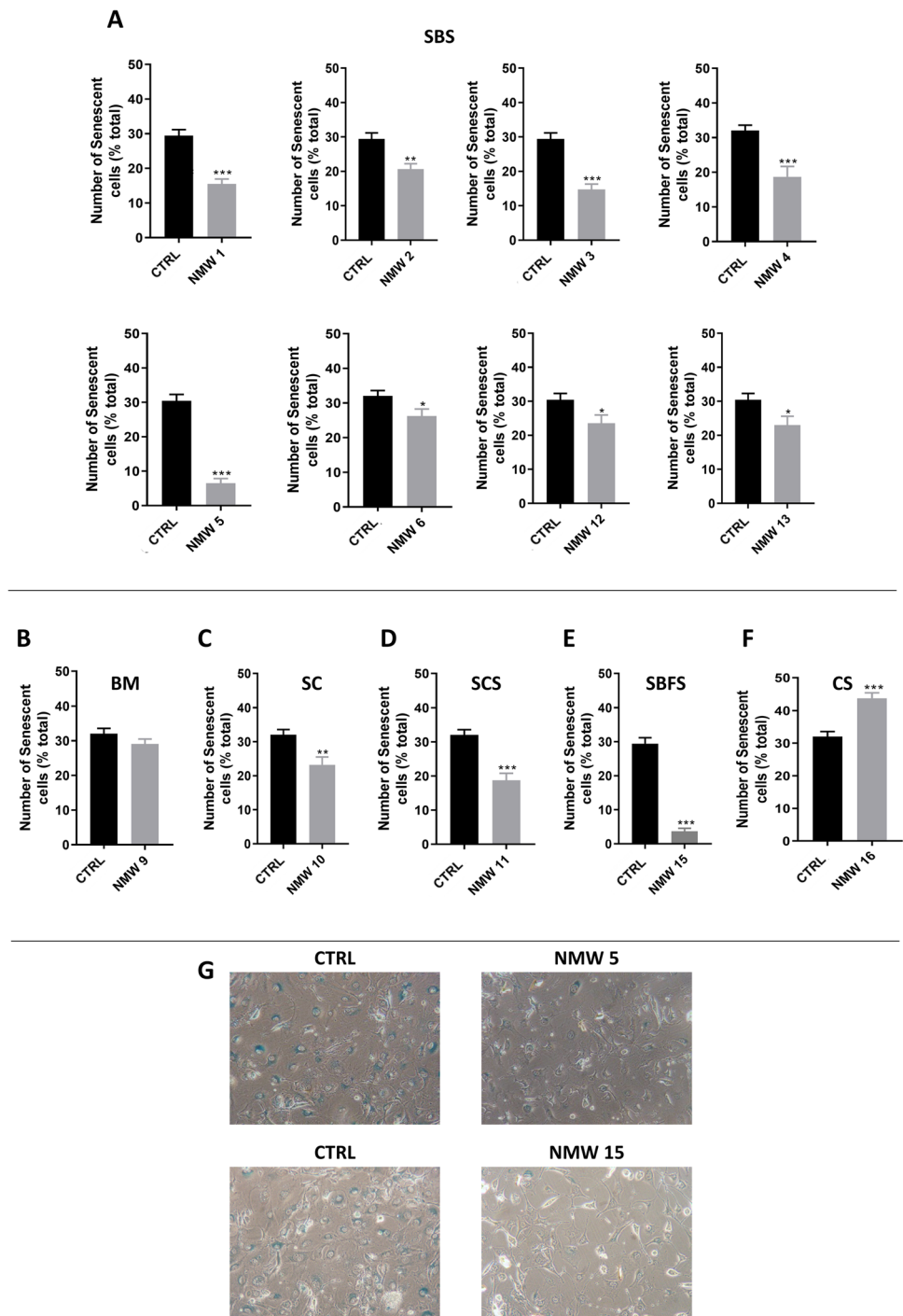
Fig. 1 Effect of natural mineral waters (NMWs) on fibroblast viability. Fibroblasts were cultured in medium prepared with NMW or with ultrapure water (CTRL). The percentage of viable cells after exposure to SBS (A), BM (B), SC (C), SCS (D), SBFS (E), and CS (F) NMW for 24 h, was evaluated by the MTT assay. Results are expressed as percentage of control group. Error bars indicate mean ± SEM of at

least three independent experiments. * $p < 0.05$ and ** $p < 0.01$ when compared with the control group. Legend: SBS, sulfurous/bicarbonate/sodic; BM, bicarbonate/magnesium; SC, sulfated/calcic; SCS, sulfurous/chlorinated/sodic; SBFS, sulfurous/bicarbonate/fluoridated/sodic; CS, chlorinated/sodic

compared with the control group (Fig. 2A). No changes were detected in cells exposed to BM NMW 9 after senescence induction (Fig. 2B). SC NMW 10, SCS NMW 11, and SBFS NMW 15 significantly abolished cellular senescence

induced by etoposide in comparison with the control group (Fig. 2C, D, and E, respectively). CS NMW 16 stimulated the etoposide-induced senescence in fibroblasts, comparatively with the control condition (Fig. 2F). An enlarged cell

Fig. 2 Effect of natural mineral waters (NMWs) on skin fibroblast senescence. Senescence-associated beta-galactosidase staining (blue color) was evaluated after exposure to etoposide for 24 h followed by SBS (A), BM (B), SC (C), SCS (D), SBFS (E), and CS (F) NMW treatment for 72 h. The percentage of beta-galactosidase positive cells was quantified in the different experimental groups by cell counting. Representative images of senescence-associated beta-galactosidase staining (blue) is presented in panel G. Magnification used was $100\times(10\times 10)$. Error bars indicate mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with the control group. Legend: SBS, sulfurous/bicarbonate/sodic; BM, bicarbonate/magnesium; SC, sulfated/calcic; SCS, sulfurous/chlorinated/sodic; SBFS, sulfurous/bicarbonate/fluoridated/sodic; CS, chlorinated/sodic



morphology and blue staining were observed in senescent cells (Fig. 2G).

The impact of NMWs 1 and 3 with the SBS ionic profile to increase viability and decrease senescence in skin fibroblasts is more relevant in comparison with other NMWs. Considering these findings, and the indication by the Portuguese Health authorities for the usage of NMW 2 for skin afflictions, we pursue the assays with NMWs 1, 2, and 3.

Effect of NMWs on cell migration and phototoxicity of murine skin fibroblasts and on anti-oxidant activity of murine macrophages

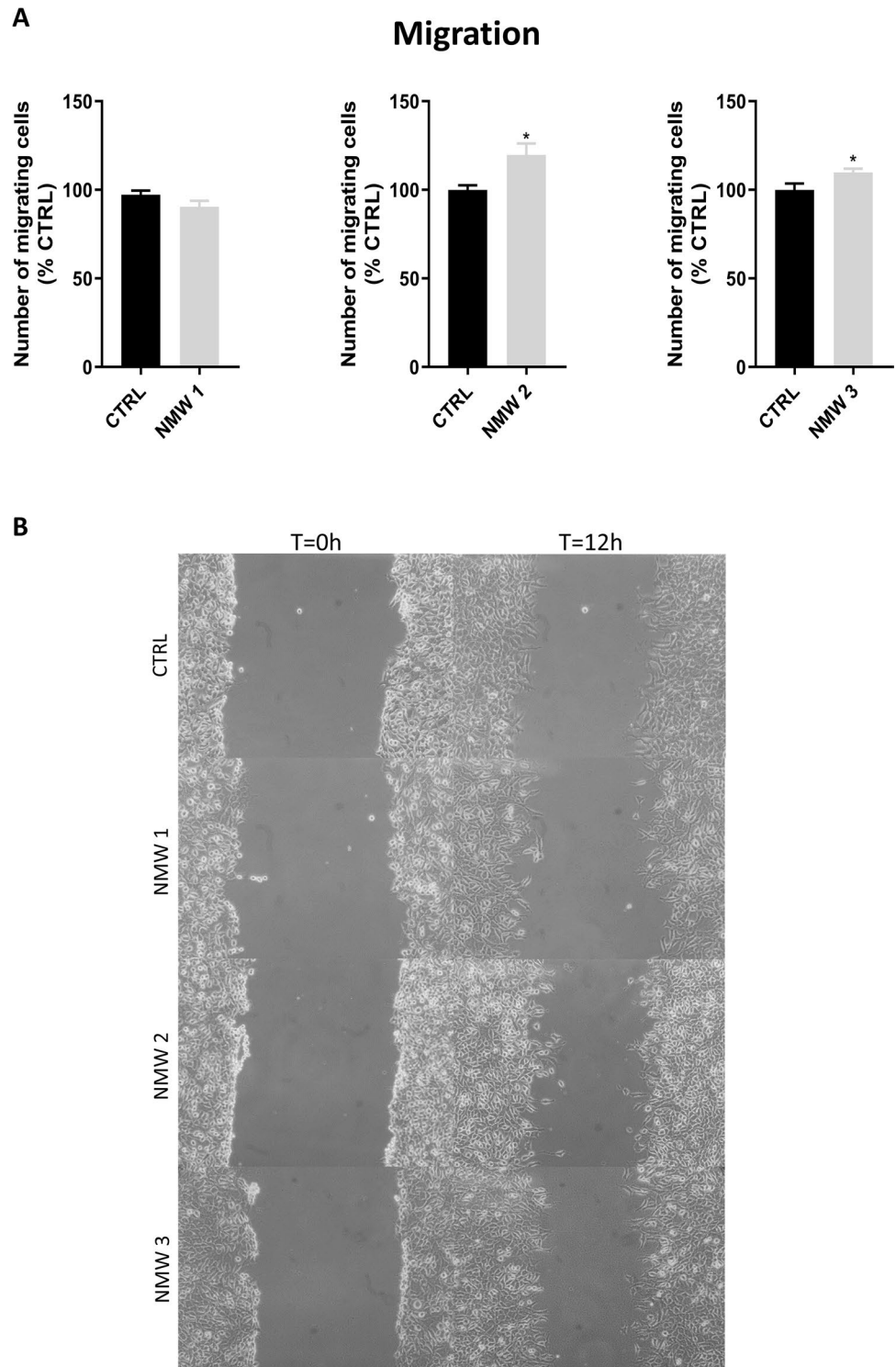
NMWs with a SBS ionic profile have an impact on the migration of skin fibroblasts

Fibroblast migration after treatment with the selected waters, specifically NMWs 1, 2, or 3 (from the SBS ionic profile),

was assessed. SBS NMW 1 demonstrated a slight, non-significant decrease on fibroblast migration, when compared with the control condition (Fig. 3A). On the other hand, fibroblast migration was significantly increased by exposure to the NMWs 2 and 3, comparatively to the control

(Fig. 3A). Representative microscopy images of migrated cells also demonstrated the effect of NMWs 2 and 3 in increasing cell migration, whereas no effect was verified with NMW 1 when compared with the control, as visually presented in Fig. 3B.

Fig. 3 Effect of sulfurous/bicarbonate/sodic natural mineral waters (NMWs 1, 2, and 3) on fibroblast migration. Cells were exposed to SBS NMWs during 12 h and cell migration was evaluated by a scratch assay (A). Representative images of migrated cells are presented in panel B. Magnification used was 100×(10×10). Error bars indicate mean ± SEM. **p* < 0.05 when compared with the control group



SBS NMWs do not present phototoxic effects on irradiated skin fibroblasts

Phototoxicity of irradiated 3T3 fibroblasts exposed to NMWs 1, 2 and 3 was evaluated. Cells treated with UVA radiation presented a decrease in cellular viability when compared with the non-irradiated group (Fig. 4).

No toxicity was observed in both irradiated and non-irradiated groups, treated with the NMW, when compared with their respective control groups.

SBS NMWs exhibit anti-oxidant properties in macrophages

In order to evaluate the *in vitro* anti-oxidant potential of SBS NMW, ROS accumulation induced by LPS exposure and the activity of the anti-oxidant enzyme superoxide dismutase (SOD) were investigated (Fig. 5).

LPS treatment induced a significantly increase on ROS production, when compared with untreated cells. NMWs 1 and 3 did not significantly affect ROS formation when compared with the control, both in basal and stimulation conditions (Fig. 5A). When the effect of SBS NMW 2 per se was evaluated, a slight but significant increase in ROS production was observed in macrophages, in the group non-stimulated with LPS relatively to control, whereas no alterations were detected between CTRL and NMW 2 LPS-treated cells (Fig. 5A).

Concerning SOD activity, the control group presented an increase when treated with LPS (Fig. 5B). Treatment of

macrophages with the SBS NMWs 1, 2, and 3, in the presence or in the absence of the LPS stimuli, activated the SOD anti-oxidant enzyme when compared with control groups (Fig. 5B).

Discussion

Different NMWs, with diverse origins and chemical fingerprints, have been studied regarding their potential to be used as cosmetic ingredients for anti-aging applications (Tacheau et al. 2018; Salsberg et al. 2019). Considering the use of Portuguese NMW on dermatological afflictions, recognized in folk medicine and by medical hydrology, there is still few scientific evidence supporting the use of these waters as valuable assets for clinical application, specifically regarding their modulatory effect of skin aging-related hallmarks (Oliveira et al. 2019).

In this study, we evaluated, for the first time, the potential of several Portuguese NMWs, with different chemical profiles, to modulate intrinsic molecular mechanisms that are closely related with skin aging. We divided the work in two major steps and started to evaluate the *in vitro* effect of the Portuguese NMW on the viability and senescence of skin fibroblasts. Then, the most promising NMW were selected to pursue in additional studies in skin fibroblasts and macrophages, which included cellular migration, photoprotection, and oxidative stress modulation.

Skin aging is a complex and multifactorial event that combines the cumulative effects of chronological aging and

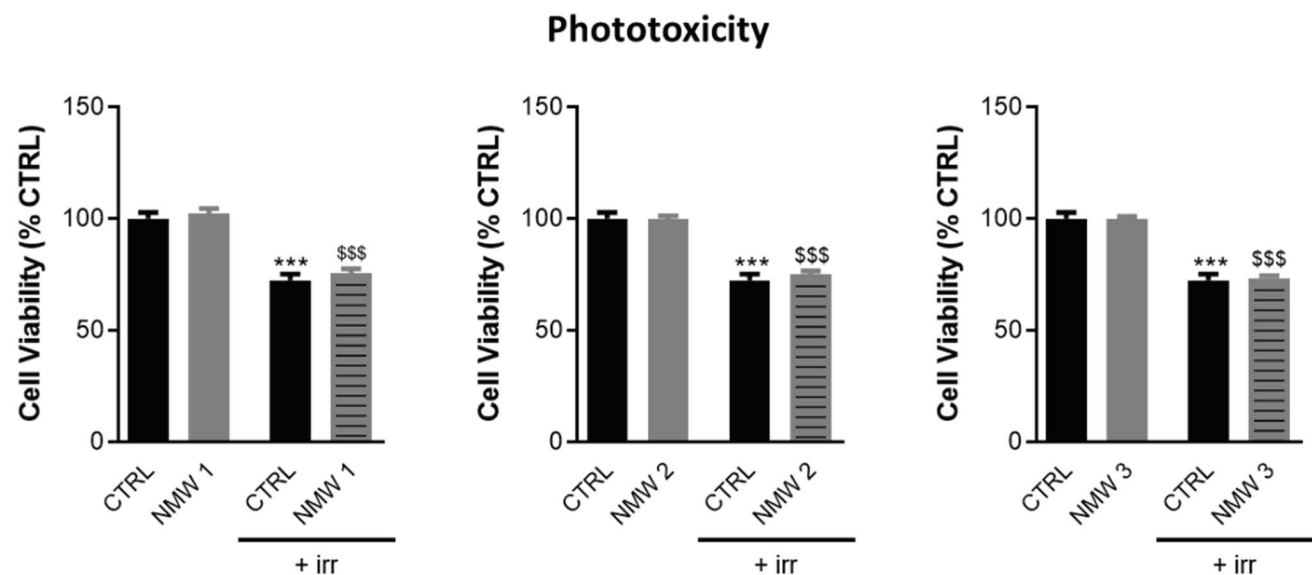


Fig. 4 Effect of sulfurous/bicarbonate/sodic (SBS) natural mineral waters (NMWs 1, 2, and 3) on phototoxicity in skin fibroblasts. The percentage of viable cells in the absence or presence of irradiation (+ irr), and exposure to SBS NMWs was evaluated spectrophotometrically using neutral red. Results are expressed as percentage of

control group without irradiation (CTRL, medium prepared with ultrapure water). Error bars indicate mean \pm SEM of at least three independent experiments. *** p < 0.001 when compared with the control group. \$\$\$ p < 0.001 when compared with NMW

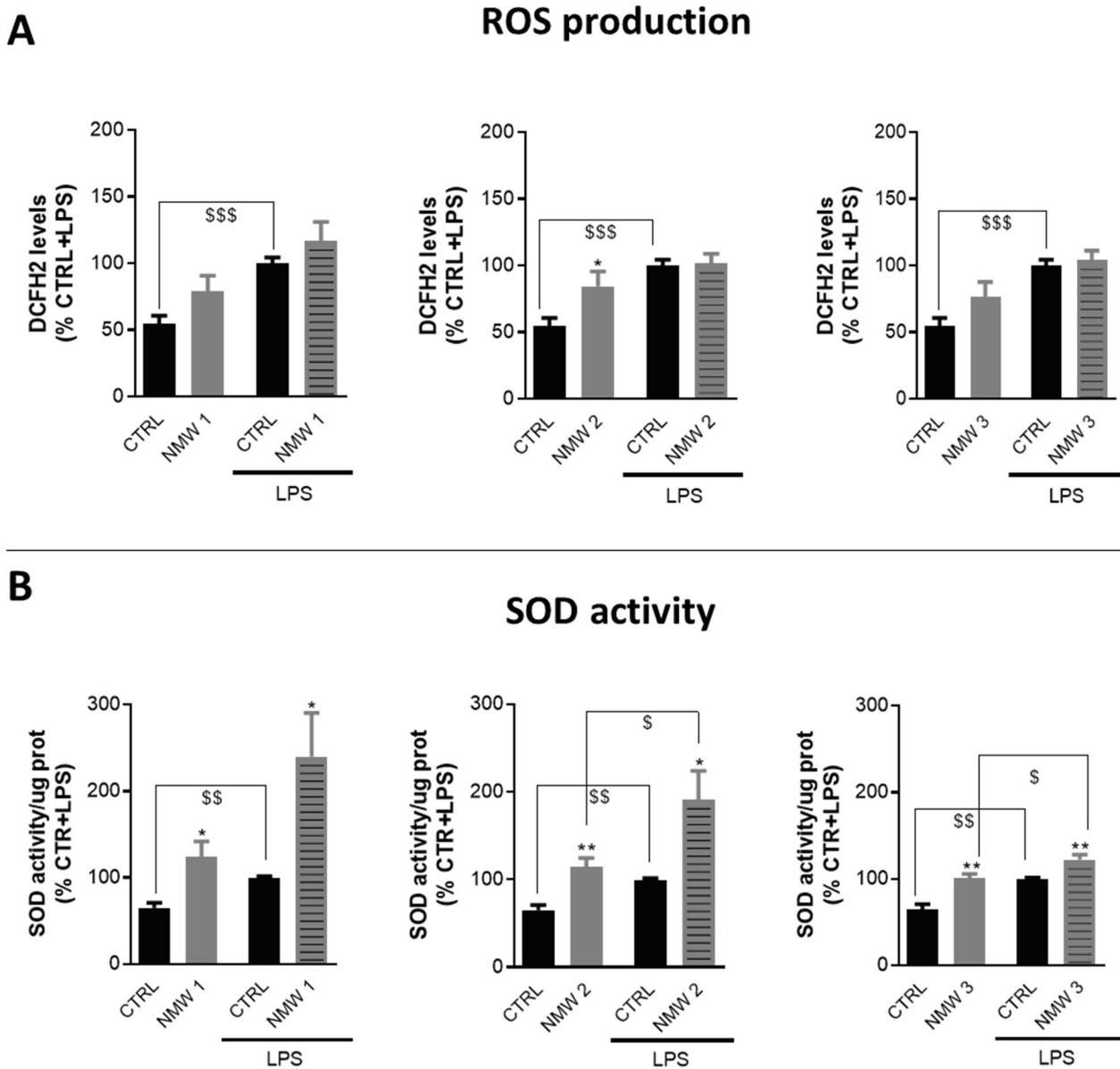


Fig. 5 Role of natural mineral waters (NMWs) on ROS production (A) and SOD activity (B) in macrophages. These innate immune cells were exposed to SBS NMW in the presence or absence of LPS (1 µg/mL), and the ROS accumulation was quantified using the 2',7'-dichlorofluorescein diacetate (H₂DCFDA) fluorescent probe or analysis of the SOD activity. Data correspond to the means ± SEM

of at least three independent experiments and are represented as % of control (CTRL) cells exposed to LPS. **p* < 0.05 and ***p* < 0.01 when compared with the respective CTRL group. \$*p* < 0.05, \$\$*p* < 0.01, and \$\$\$*p* < 0.001 when compared with the respective group without LPS. Legend: SBS, sulfurous/bicarbonate/sodic

environmental factors (Tacheau et al. 2018). The development of skin aging, as well as many skin diseases, results from the loss of cell homeostasis, in which dysfunctional replication can result in senescent cells (Ganceviciene et al. 2012; Wang and Dreesen 2018). Fibroblasts are the major cell type in the dermis, involved in skin injury recovery, and a loss of their activity seems to be a crucial factor for skin aging (Wang and Dreesen 2018; Lämmermann et al. 2018). Therefore, in the present study, we first investigated

the in vitro effect of several Portuguese NMWs on the cell viability and senescence of fibroblasts. As disclosed in the “Results” section, NMWs 1 and 3 from the SBS group were able to significantly increase viability and decrease senescence of fibroblasts. Additionally, all SBS NMWs studied prevented senescence induced by treatment with etoposide, a well-known inducer of cellular senescence (Biran et al. 2017; Teng et al. 2021). The mitigation of cellular senescence was also triggered by NMWs that exhibited different ionic

profiles, which led us to hypothesize that their effect may not be exclusively related to the ionic composition. When considering their physicochemical composition and hence their adopted classification, SBS NMWs belong to the poorly mineralized NMW group and are characterized by the presence of sulfur, bicarbonate, and sodium in their composition. In addition to these ions, they also seem to be grouped by their levels of potassium, chloride, pH, and alkalinity, as disclosed in our own previous findings (Supplementary Table S1 and Oliveira et al. 2019). Several minerals that are present in NMWs are known to have beneficial effects on skin or have important functions in different physiological activities, modulating several skin reactions. In fact, sulfur has been referenced as an element involved in cellular regeneration, with anti-oxidant and antibacterial properties (Table 1) (Coutinho et al. 2015). Sodium and potassium also contribute for cellular renewal and metabolic control (Table 1) (Nunes and Tamura 2012; Coutinho et al. 2015). Therefore, the presence of these chemical compounds in SBS NMWs may account for their capacity to prevent senescence and to promote cell viability. Regarding NMW 16, an opposite effect was demonstrated. In fact, this NMW reduced the viability of skin fibroblasts and was the only NMW that increased the percentage of senescent cells. NMW 16 belongs to the CS group and is, therefore, classified as an acidic and hyposaline water. Considering their acidic profile, we hypothesized that its distinct pH may result in a negative impact on cell viability and senescence, and therefore, an additional control with Milli-Q-type water at the NMW pH was introduced in this study. We concluded that the observed cytotoxicity was due to the lower pH (pH = 5.4) (Oliveira et al. 2019); however, it does not explain the effect on cell senescence (results not shown). Therefore, other intrinsic characteristics besides pH, such as the existence of specific compounds or osmolality values, may contribute to this decrease in cell viability and increase in cell senescence. Lee et al. described a decrease in keratinocyte viability after exposure to undiluted spring water, a reduction that seemed to be related with the water's high osmolality (Lee et al. 2012). Our own team demonstrated that another NMW with acidic pH decreases skin cell proliferation, migration, and senescence in an independent pH mode, which can be related with a higher content on silica (Oliveira et al. 2020).

Altogether, our results suggest that several Portuguese NMWs may possess interesting properties for anti-aging purposes. In order to pursue with additional studies addressing anti-aging mechanisms, we selected SBS NMW 1 and SBS NMW 3, due to their unique combination of increased viability and decreased senescence. In addition, the NMW 3 was previously demonstrated by our research team to have anti-inflammatory properties, making this NMW even more attractive to be studied for its anti-aging properties (Silva et al. 2020a). We also included one additional NMW from

the SBS chemical profile (NMW 2) that, despite not promoting an increase on cell viability, is one of the NMW regulated by the Portuguese Health authorities with indication for skin afflictions. We also take into account its complementary promising results in terms of dermocosmetic formulation and anti-inflammatory properties, previously published by our own team and others (Coutinho et al. 2015; Nunes et al. 2019; Silva et al. 2020b). Indeed, chronic low-level inflammation has been associated with aging, the so-called inflammaging. All selected NMWs belong to SBS chemical profile. Despite that different chemical profiles can be found on Portuguese NMW, sulfurous ones are the most common (Viegas et al. 2019), being the most representative in terms of Portuguese endogenous NMW resources.

Another endogenous factor associated with cutaneous aging is the loss of elasticity. Aged fibroblasts typically display changes in cell morphology with a reduction in motility and increased mechanical strength (Sliogeryte and Gavara 2019). These cells also present decreased migration and proliferation upon aging and ultimately exhibit loss of flexibility and elasticity (Sliogeryte and Gavara 2019). Furthermore, it is known that age-related senescence reduces the migration capacity of fibroblasts both *in vitro* and *in vivo* (Sliogeryte and Gavara 2019). As disclosed in the Results section, two of the three-selected SBS NMWs (NMWs 2 and 3) were able to increase migration of cultured fibroblasts, which may indicate a potentially relevant role of these NMWs on recovery after a skin insult. Other authors have reported the beneficial effect of NMWs on cell migration and proliferation. Nicoletti et al. used an Italian calcium magnesium bicarbonate-based water (Comano, Italy) in cultured skin fibroblasts and observed an increase in proliferation (Nicoletti et al. 2017). Faga et al. also used the same Comano spring water in a rabbit wound model and found that the topical application of the NMW reduced inflammatory cell infiltration with selective fibroblast recruitment (Faga et al. 2012). Also, the Comano water increased neocollagen synthesis and elastic fiber regeneration. The authors proposed that this positive effect on skin regeneration could be due to the favorable combination of a local wet environment with an anti-inflammatory action. Our study was focused on *in vitro* studies, and so, we cannot conclude about the effect of the microbiota environment. However, a previous study of our research group disclosed the anti-inflammatory capacity of the three NMWs investigated, by decreasing NO production and iNOS expression (Silva et al. 2020a), which is also a favorable condition for skin recovery. Regarding other Portuguese NMWs, our team had previously reported the beneficial effect of a silica-rich NMW in the decrease of cellular migration of skin fibroblasts, which could have a positive effect in hyper-proliferative skin conditions, such as psoriasis (Oliveira et al. 2020). These reports, combined with the effects on cellular migration obtained with NMWs

from the same chemical profile, led us to suppose that the effect may be water-specific; however, the presence of some minerals, namely calcium, magnesium, bicarbonate, and sulfur (Oliveira et al. 2019), or their combination, could be involved in cellular migration and recovery.

Furthermore, it was also evaluated the effect of the three-selected NMW regarding their phototoxicity/photo-protection, after exposure of fibroblasts to UVA radiation. This insult is classically described as a cause of photoaging through production and release of free radicals, which induce cellular damage and the expression of matrix metalloproteinases that degrade collagen and elastin (Kim et al. 2015). Altogether, these events reduce skin elasticity and increase wrinkle formation and skin dryness (Kim et al. 2015). In a recent study, Gerencsér et al. reported that some NMWs can prevent adverse effects of solar or artificial UV radiation on human skin (Gerencsér et al. 2019). Interestingly, none of our tested NMW was able to revert cytotoxicity caused by UVA irradiation. In their study, Gerencsér et al. concluded that the organic fraction of the studied NMW was, in fact, responsible for the UV photo-protection (Gerencsér et al. 2019). NMWs, in addition to relatively high amount of inorganic salts and dissolved gasses, also contain several organics in a great variety and abundance. Since our study design included the filtration of the NMW, we did not account for the possible beneficial effect of the organic fraction. This may explain, in part, the lack of positive results regarding photoprotection. However, these NMWs do not potentiate phototoxicity and therefore can be used for skin treatment, or for application in pharmaceutical formulations without problems associated with light exposure. More studies using the organic fraction would be interesting in order to evaluate the possibility of photoprotection of the NMWs.

Another important factor on skin aging is the oxidative stress that is caused by increased ROS levels and by damaging the oxidative defense system, playing a major role in the development of skin disorders, in cellular aging and/or senescence, and consequently in aging-related diseases (Rinnerthaler et al. 2015). Anti-oxidants, such as SOD and others, can interact with ROS or their products, by eliminating or minimizing their effects (Bickers and Athar 2006; Gu et al. 2020). Therefore, maintaining redox balance is a very relevant issue to be considered in the development of new anti-aging products or new therapeutics for skin diseases. Macrophages, as fibroblasts and keratinocytes, are involved in the maintenance of skin organ equilibrium and have been routinely used as an *in vitro* model to study oxidative stress (Park et al. 2011; Su et al. 2011). Consequently, our next step focused on the evaluation of ROS generation and anti-oxidant SOD activity in macrophages under oxidative stress conditions, after exposure to the selected NMWs. All three selected NMWs were able to control oxidative stress in macrophages, by activating the anti-oxidant enzyme SOD

in response to ROS induction. Other studies using different NMWs have reported an anti-oxidant capacity, evoked from different mechanisms (Hercogova et al. 2002; J. Richard et al. 2010; Bieber 2011; Nicoletti et al. 2016). La Roche-Posay (France) NMW, naturally rich in selenium, has shown an increase in fibroblast survival after the exposure to free radicals. Additionally, higher levels of SOD were present after cell exposure to La Roche-Posay water, enhancing its anti-oxidant activity (J. Richard et al. 2010). It has also been shown that Avène thermal water (France), a bicarbonate and silicate water with high content of calcium and magnesium, presents anti-oxidant activity by reducing lipid peroxidation in human keratinocytes (Joly et al. 2000; Merial-Kieny et al. 2011). Herein, the selected NMWs are all bicarbonated, similar to the waters described in the literature as having anti-oxidant properties. Also, SBS NMWs are rich in sulfur, an element well known for its anti-oxidant properties, and its compounds are involved in multiple, complex anti-oxidant mechanisms, including ROS scavenging, glutathione peroxidase activity, and metal binding (Table 1) (Battin and Brumaghim 2009; Coutinho et al. 2015).

Conclusion

Treatment of dermatological diseases and the relief of their associated symptoms, along with the development of anti-aging products, still present a major challenge to medical and pharmaceutical industries. The evaluation of the cytoprotective, anti-senescence, and anti-oxidant effects of NMW, and their correlation with the waters' composition, may scientifically support the effect of NMWs in skin aging-related processes. By using *in vitro* cell models, the NMW's effect may be evaluated in a non-biased mode, as the mechanism behind their beneficial effects *in vivo* can be related to several uncontrolled factors, as the smoothing environment of the thermal centers in which the treatments are performed.

Skin aging is a complex process, produced by a combination of intrinsic cellular mechanisms and extrinsic factors. Therefore, NMWs that have the ability to regulate different intrinsic cellular mechanisms may be important resources with anti-aging potential.

SBS NMWs that were able to control oxidative stress were also effective in diminishing fibroblast cell senescence, increasing viability and migration, which are crucial and inter-related factors in skin cell regeneration and aging. Different chemical elements that are present in these waters, namely sulfur, bicarbonate, sodium, potassium, and silica, seem to be related with the waters' activity in skin cells. Importantly, the mechanism behind the NMWs effect may result from the combination of the different elements and need future clarification. As referred by other authors, each NMW has to be evaluated

as a whole, or a combination of several elements, instead of focusing on the contribution of a specific element.

Despite that only a few thermal centers are legislated for dermatological applications, namely for seborrhea, acne, eczema and psoriasis, the presented in vitro findings represent the starting point to scientifically support the role of SBS NMWs as promoters of skin health with a special focus on skin aging. With this in vitro study, we ultimately conclude that the use of SBS NMW directly or formulated as cosmetics or medical devices could be of value for an anti-aging application and/or symptom alleviation of different skin conditions, and further research on this matter should be pursued.

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