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ORIGINAL PAPER

In vitro evaluation of potential benefits of a silica-rich thermal water (Monfortinho Thermal Water) in hyperkeratotic skin conditions

Ana Sofia Oliveira[1] · Cátia Vicente Vaz[1] · Ana Silva[2] · Sara Correia[1] · Raquel Ferreira[1,3] · Luiza Breitenfeld[1,3] · José Martinez-de-Oliveira[1,3] · Rita Palmeira-de-Oliveira[1,2,4] · Cláudia Pereira[2,5] · Maria Teresa Cruz[2,6] · Ana Palmeira-de-Oliveira[1,3,4]

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Abstract

Thermal therapy has gained popularity over the years, and Portugal is one of the richest European countries in mineral therapeutic waters. The interest in the use of these natural mineral waters (NMW) for dermatologic purposes is continuously growing but there is a lack of scientific studies supporting its health benefits. The study aims to investigate the effect of a silica-rich NMW in skin cell homeostasis using two representative cell lines of the epidermis and dermis, keratinocytes and fibroblasts, respectively, in addition to a macrophage cell line. Mouse skin fibroblasts, macrophages and human keratinocytes were exposed to culture medium prepared with NMW. Cell metabolism (MTT or resazurin assays) and cell proliferation (trypan blue exclusion dye assay) were investigated. Migration (scratch-wound assay) and senescence (β-galactosidase activity assay) of fibroblasts were also studied. Exposure to NMW compromised the cell metabolic state of all the cell lines tested. This impairment was more pronounced in skin keratinocytes (60% reduction) relatively to skin fibroblasts (45% reduction) or macrophages (25% reduction). Proliferation of macrophages was reduced threefold upon exposure to thermal water, compared to controls. No differences were observed in migration between fibroblasts exposed to NMW and controls, while a potentiation of senescence of these cells was observed. Our results shed light in the bioactive effects of a silica-rich NMW supporting its therapeutic use. A reduction in both cell metabolism and proliferation of keratinocytes and macrophages supports the empirical clinical benefits of this NMW in hyperkeratotic conditions, such as psoriasis and atopic dermatitis.

Keywords Balneology · Thermal water · Keratinocytes · Macrophages · Cell proliferation · Psoriasis

✉ Ana Palmeira-de-Oliveira
apo@fcsaude.ubi.pt

[1] Health Sciences Research Centre (CICS-UBI), University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal

[2] Center for Neuroscience and Cell Biology, University of Coimbra, Rua Larga, 3004-504 Coimbra, Portugal

[3] Faculty of Health Sciences, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal

[4] Labfit–Health Products Research and Development Lda, UBImedical, Estrada Nacional 506, 6200-284 Covilhã, Portugal

[5] Faculty of Medicine, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

[6] Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

Introduction

Natural mineral (or thermal) waters (NMW) are natural solutions that originate from the subsoil under specific geological conditions, present 'physicochemical dynamism' and share three fundamental characteristics: originate naturally from the earth as 'springs', are bacteriologically pure and have some therapeutic potential or health beneficial effect (Matz et al. 2003; Ferreira et al. 2010; Araujo et al. 2017). In fact, to be characterized as a NMW, the following features have to be addressed:

- Geological characteristics, including a detailed description of the collection site, the nature of the terrain, the stratigraphy of the hydrogeological layer and a description of the collection procedures;

- Physical, chemical and physicochemical properties, including the main physical and chemical analysis to detail the final characteristics of the mineral water;
- Microbiological fingerprint, ensuring the absence of the main contamination indicators (parasites and pathogenic microorganisms, *Escherichia coli* and fecal streptococci, sporulated sulphite-reducing anaerobes, *Pseudomonas aeruginosa*);
- Possible pharmacological, physiological and clinical effects (Quattrini et al. 2016).

The health benefits of NMW were widely recognized long before modern medical treatment development, and balneotherapy, which consists on the use of thermal mineral waters, mud/peloid packs, natural gases or hay baths for preventive, therapeutic and rehabilitative purposes, is considered an interesting alternative treatment for several conditions (Hercogova et al. 2002; Antonelli et al. 2018; Gerencsér et al. 2019). In fact, in the last years, many thermal centres have been increasingly requested for the relief of numerous afflictions ranging from neuromuscular, gastrointestinal, epidermic/dermic to articular and respiratory disorders (Gomes et al. 2010).

The dermatological therapeutic indication of NMW is well established in different countries. One important example is the application of NMW in the French cosmetic industry due to their recognized biological effects in different skin conditions (Nunes and Tamura 2012). The major skin conditions associated with balneotherapy with a high rate of success are psoriasis and atopic dermatitis (AD) (Matz et al. 2003). Psoriasis is a chronic autoimmune disease that affects approximately 3% of the population worldwide and is characterized by hyper-proliferation and altered differentiation of keratinocytes, as well as the infiltration of immune cells into lesioned skin (Leite Dantas et al. 2016; Benhadou et al. 2019). AD is also a common chronic skin disease characterized by hyperkeratosis of epidermis and fibrosis within the dermis, concomitantly with recurrent pruritic inflammatory skin lesions resulting from a compromised skin barrier (Aries et al. 2016).

Despite the high demand of NMW for the relief of dermatological conditions, the efficacy of thermal therapy for the treatment of skin diseases and the knowledge of the mechanisms underlying its bioactivity are only partly disclosed, and probably combine chemical, thermal, mechanical and immunomodulatory effects (Türsen 2019). One of the most important factors that support the therapeutic effects of NMWs is the physicochemical composition (Coutinho et al. 2015). In fact, the dermatologic therapeutic indications for NMW are strongly related to the presence specific elements namely sulfur, silica and different cations, such as sodium, calcium and potassium (Araujo et al. 2017). In addition to the classical putative role of chemical elements, recent studies have also described the possible beneficial influence of NMWs' native microbiome (Varga 2019; Nicoletti et al. 2019).

Portugal has an old tradition of using NMW as traditional medicine and there are about 50 thermal centres nationwide (Coutinho et al. 2015; Rebelo et al. 2015; Oliveira et al. 2019). The majority of therapeutic indications of Portuguese NMWs are for respiratory, rheumatic and musculoskeletal problems. Still, about 50% of thermal centres, present in national territory, have recognized dermatological therapeutic indications (Diário da República 1989). One of Portuguese thermal centres with a highly recognized therapeutic indication for skin conditions is the Monfortinho thermal centre (Diário da República 2008) . Regarding total mineralization and main chemical components, Monfortinho NMW is characterized as hyposaline (< 200 mg/L), as defined by the Institute of Hydrology of Lisbon (Cantista 2008) and classified as bicarbonated, with high concentration in calcium, magnesium, sodium and silica. Sodium and silica represent more than 50% of total mineralization, which may justify the NMW dermatological activity, since sodium and silica are described to contribute to a rapid recovery from skin injuries (Araujo et al. 2017; Almeida et al. 2019).

Recently, the skin beneficial properties of this silica-rich NMW were evaluated by studying the effect of creams formulated with NMW on skin hydration and treatment of psoriasis and eczema (Almeida et al. 2019). In this study, no differences were observed when NMW was used compared to ultra-pure water. Despite these results, beneficial effects of the use of this NMW are undoubtedly acknowledge by people who seek the thermal centre. Therefore, a more fundamental investigation is needed in order to scientifically sustain the empirical beneficial effects of this NMW.

In this study, we used representative cell lines of different skin layers namely keratinocytes (epidermis) and skin fibroblasts (dermis) to study the effects of a silica-rich NMW in cellular response, particularly on elementary mechanisms involved in skin homeostasis, namely cellular metabolism, proliferation, migration and senescence.

Additionally, we included a mouse macrophage cell line, to address the effect of the NMW on cellular metabolism and proliferation of innate immune cells, which also have an important role in skin conditions, thus allowing a broad characterization of biological activities of this NMW.

Methods

Natural mineral water collection

NMW was collected in Monfortinho Thermal Centre from the spring/borehole AC7, in Portugal. One sample, divided into six appropriate flasks (VWR, Pennsylvania, USA), was collected following appropriate water collection procedures

(APHA 2005). The collected water sample was sealed and placed in thermal boxes and transported to the laboratory under the recommended conditions (5 ± 3 °C) and kept closed and refrigerated in the dark until use.

Physicochemical parameters

Upon arrival at the laboratory, a microbiological quality control test, colony-forming unit (CFU) count was performed in Sabouraud Dextrose Agar and Tryptic Soy Agar (both from VWR, Pennsylvania, USA), to address the presence of fungus and bacteria, respectively, as described (INFARMED - Instituto Nacional da Farmácia e do Medicamento 2009). If microbial growth was present, the water samples were filtered through a 0.2-μm pore filter (VWR, Pennsylvania, USA), to ensure the necessary sterility for the remaining assays.

Physicochemical parameters specifically odour, aspect, colour, deposit, osmolality and pH were analysed using internal methods based on Standard Methods for the Examination of Water and Wastewater (SMEWW) (APHA 2005). All measurements were performed in triplicate at room temperature (±25 °C).

Other relevant physicochemical analyses were performed in an independent laboratory accredited by the NP EN ISO / IEC 17025 norm.

Cell culture

Cell lines

Mouse leukemic monocyte macrophage cell line RAW 264.7 (ATCC Cat# TIB-71, RRID:CVCL_0493) were cultured in DMEM medium pH 7.4, supplemented with 10% foetal bovine serum (FBS) (both from Life technologies, California, USA) and 25 mM glucose, 17.95 mM sodium bicarbonate, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Sigma-Aldrich, Missouri, USA).

Mouse skin fibroblasts cell line 3T3 (ATCC Cat# CRL-1658, RRID:CVCL_0594) and human keratinocytes cell line HaCaT (CLS Cat# 300493/p800_HaCaT, RRID:CVCL_0038) were grown in DMEM medium supplemented with 10% heat inactivated FBS (both from Life Technologies, California, USA) and 25 mM glucose, 35.9 mM sodium bicarbonate, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Sigma-Aldrich, Missouri, USA).

Cells were kept at 37 °C in 5% CO_2 humidified atmosphere. Sub-culturing was performed according to the manufacturer's recommendations. Morphological cell alterations were monitored by microscope observation.

Cellular stimulus using NMW

NMW was used undiluted in the culture medium preparation, instead of MilliQ-type water, to expose the cells to NMW as described elsewhere (Nicoletti et al. 2017). The final pH was adjusted to the intrinsic NMW pH in order to maintain the water's characteristics. MilliQ-type water was used to prepare control culture medium (control pH = 7.4). An additional control, MilliQ-type water with the pH adjusted to intrinsic NMW pH (5.6) was used do discard a possible effect of the water pH itself. To corroborate this hypothesis, a second pH control was applied, when necessary, consisting of NMW with pH adjusted to a physiological condition (pH = 7.4). Cells were exposed to undiluted NMW or to different controls for 12 h for migration assays, 24 h and 48 h for cellular viability and proliferation screenings and 72 h for evaluation of induced senescence.

Cellular metabolic activity screening

Metabolic activity of macrophages was assessed using resazurin reduction assay, as previously described (O'Brien et al. 2000). Briefly, cells were plated in a density of 2.5×10^5 cells/mL, in a 96-well plate (200 μl/well), and exposed to NMW for 24 or 48 h. Resazurin (Sigma-Aldrich, Missouri, USA) solution prepared with sterile phosphate buffer solution (50 μM) was added to each well after NMW exposure. After 4 h, absorbance was read at 570 and 620 nm with a xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad, California, USA).

Due to the slow rate of resazurin conversion of mouse skin fibroblasts and human skin keratinocytes, the metabolic activity of these cells treated with NMW was evaluated by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Alfa Aesar, Massachusetts, USA) assay, as described elsewhere (Moura et al. 2013).

Briefly, 5×10^4 cells/mL (fibroblasts) and 1×10^5 cells/mL (keratinocytes) were seeded in a 96-well plate (200 μl/well) and, after 24 h, were exposed to Monfortinho NMW for 24 or 48 h. Thereafter, MTT solution (in phosphate buffered saline) was added to each well (final concentration − 0.5 mg/mL) and the plates were incubated at 37 °C for 4 h. The MTT-containing medium was discarded and dimethyl sulfoxide (Sigma-Aldrich, Missouri, USA) was added to dissolve the formazan crystals. Formazan quantification was performed using the xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad, California, USA) at 590 nm, with a reference wavelength of 620 nm.

For the different cell lines, cell metabolic activity results were expressed as a percentage of control prepared with MilliQ-type water at pH 7.4 (untreated cells).

Cellular proliferation screening (trypan blue dye exclusion assay)

Cellular proliferation was addressed as previously described (Moura et al. 2013). Cells were plated in a density of 5×10^4 cells/mL (fibroblasts) and 2.5×10^5 cells/mL (macrophages), in a 6-well plate (2 mL/well), and exposed to NMW and controls, for 24 or 48 h. Cells were detached using Trypsin–EDTA 0.5%/0.2% EDTA (Sigma-Aldrich, Missouri, USA) or a cell scrapper (fibroblasts and macrophages, respectively). Then, a trypan blue solution 0.4% (w/v) (AMRESCO Inc., Ohio, USA) was added to a cell suspension aliquot (1:1 ratio), and the number of viable and dead cells (giving the total cell number) was counted manually using a Neubauer chamber under a microscope (Olympus CK40).

Fibroblasts migration assay

Cell migration was evaluated using a scratch-wound assay, as described elsewhere with modifications (Ferreira et al. 2012). Before fibroblasts were plated (2.5×10^5 cells/mL in a 12-well plate), two parallel lines were carved on the underside of each well with a scalpel to serve as a guidance axis. When cells were approximately 95% confluent, a wound was made by a perpendicular scratch with a P10 pipette tip and photographs were taken at this time ($t = 0$) using an inverted microscope (Olympus CKX41) with a ×10 objective and an Olympus digital camera (DC 6 V). After, cells were exposed with medium prepared with NMW, and respective controls, using 2% FBS (v/v) to reduce the proliferation rate during the experiment. After 12 h, the same area was photographed. Images were segmented through machine learning and Python analysis integrated in the Zeiss Zen Intellesis software (ZEN Digital Imaging for Light Microscopy, RRID:SCR_013672). A map of the different regions on each image was obtained and the area corresponding to the free-cell region was then quantified using FIJI software (Fiji, RRID:SCR_002285).

Senescence-associated β-galactosidase in fibroblasts

Senescence was assessed using a commercially available beta-galactosidase staining kit according to the manufacturer's protocol (Cell Signaling Technology, Massachusetts, USA). Briefly, 2.5×10^4 fibroblasts cells/mL were plated in 12-well plates and allowed to adhere overnight. Senescence was induced with 12.5 μM etoposide for 24 h. The senescent agent was removed and cells were washed. Thereafter, the cells were allowed to recover in NMW or in standard media (control) during 72 h. Cells were then fixed and incubated overnight with beta-galactosidase staining solution in a dry incubator at 37 °C without CO_2 supply to prevent false positivity. Wells were inspected under microscope for blue colour development and were photographed for image analysis (8 random fields per condition). A distinct blue colour staining is indicative of beta-galactosidase activity. Quantitative analysis was carried out using ImageJ (ImageJ, RRID:SCR_003070).

Statistical analysis

Cellular assays were carried out in triplicate in three independent experiments, and all values were expressed as percentages of the control prepared with MilliQ-type water. One-way ANOVA with Tukey's multiple comparisons test was performed to compare NMW with the other conditions. p value <0.05 was accepted as denoting statistical significance. All analyses were conducted using GraphPad Prism version 7.03 for Windows (GraphPad Prism, RRID:SCR_002798).

Results

Physicochemical parameters

To obtain a complete physicochemical characterization of Monfortinho NMW, the parameters measured in our laboratory were complemented with detailed information disclosed in the analytical report provided by an independent laboratory. As shown in Table 1, Monfortinho NMW presented no odour, colour or deposit, exhibiting a limpid aspect, and an acidic pH of 5.6. Of note, this NMW presented a 'total mineralization' value of 57.2 mg/L and a high silica value of 23.7 mg SiO_2/L being a hypo-saline and silicate water, as defined by Cantista (2008). Regarding its content in anions and cations, the most prevalent ion is silicate (37.5 mg $H_3SiO_4^-$/L) followed by bicarbonate (9.86 mg HCO_3^-/L). It is important to note that carbonate (CO_3^{2-}) was not detected in this NMW. Also, results for ions chloride (Cl^-), nitrates (NO_3^-), nitrites (NO_2^-) and ammonium (NH_4^+) and for physicochemical constants conductivity and total alkalinity, disclosed in the analytical sheet, were below the quantification limit of the method used (not shown).

At arrival, the water sample presented a positive result in the microbiological control with 2 and 4 CFU/ml for bacteria and fungus, respectively, hence the water was firstly filtrated prior to its use in cellular assays.

Cellular metabolic activity screening

The ability of NMW to influence cellular metabolic activity was screened using either MTT (keratinocytes and fibroblasts) or resazurin (macrophages).

As represented in Fig. 1a–f, cell exposure to MNW for 24 or 48 h caused an overall significant decrease in metabolic activity in the different cell lines, when compared with

Table 1 Physicochemical parameters of Monfortinho NMW

Physicochemical composition	Result
Organoleptic features	
Odour†	No odour
Deposit†	Null
Aspect†	Clear
Colour†	Without colour
Physicochemical constants and non-dissociated substances	
Temperature emergency (°C)	28.6
pH (at 25 °C) †	5.6
Osmolality (mOsmol/kg)	36
Total hardness (mg $CaCO_3$/L)	9
Silica (mg SiO_2/L)	23.7
Dry residue at 180 °C (mg/L)	42
Total mineralization (mg/L)	57.2
Microbiological control	
Bacterial (CFU/mL)	2
Fungal (CFU/mL)	4
Anions and cations	
Silicate (mg $H_3SiO_4^-$/L)	37.5
Bicarbonate (mg HCO_3^-/L)	9.86
Sodium (mg Na^+/L)	2.61
Magnesium (mg Mg^{2+}/L)	1.48
Sulphate (mg SO_4^{2-}/L)	1.33
Calcium (mg Ca^{2+}/L)	1.32
Potassium (mg K^+/L)	0.69
Iron (mg Fe^{2+}/L)	0.005
Lithium (mg Li^+/L)	0.0045
Fluoride (mg F^-/L)	0.04

† Represents measurements performed in our laboratory upon arrival of the water samples

control, in the absence of NMW. In general, there were no statistical significant differences between cells exposed to NMW and the other experimental conditions designed to control pH.

Regarding the skin representative cell lines, a relevant effect in cell metabolic activity was observed 24 and 48 h after NMW treatment. In keratinocytes, the compromise in metabolic activity was more pronounced, with a decrease of approximately 60% when compared to the control. Metabolic impairment was also evident in fibroblasts with a decrease of 45% after exposure to NMW. In both cell lines, no difference was observed in the decrease in cell metabolism when cells were exposed to MilliQ-type water at pH 5.6 (Control pH = 5.6) compared to control. Also, no difference was detected when comparing NMW at its intrinsic pH with a physiological adjusted pH.

Concerning the macrophage cell line, a significant decrease on cell metabolism was also found, however, in a lower extent, specifically ranging from 15 to 25% when compared to the control, after 24 and 48 h of exposure, respectively. Upon 48-h exposure to NMW and MilliQ-type water at pH 5.6 (control pH = 5.6), a statistical significant difference in the metabolic activity was observed between these two conditions.

Cellular proliferation

The ability of NMW to influence cell proliferation was also evaluated in cell lines where a metabolic impairment did not achieved 50% (fibroblasts and macrophages) in order to evaluate the effect of cell proliferation in the overall cell viability. Contrarily to what happened in cell metabolism, significant alterations regarding cell proliferation were only present after 48 h exposure to NMW (Fig. 2a, b).

In fibroblasts, a moderate decrease in cell proliferation was detected, which is in accordance with metabolic activity. Regarding macrophages, a more pronounced decrease in cell proliferation was present (threefold decrease when compared with the control, Fig. 2b).

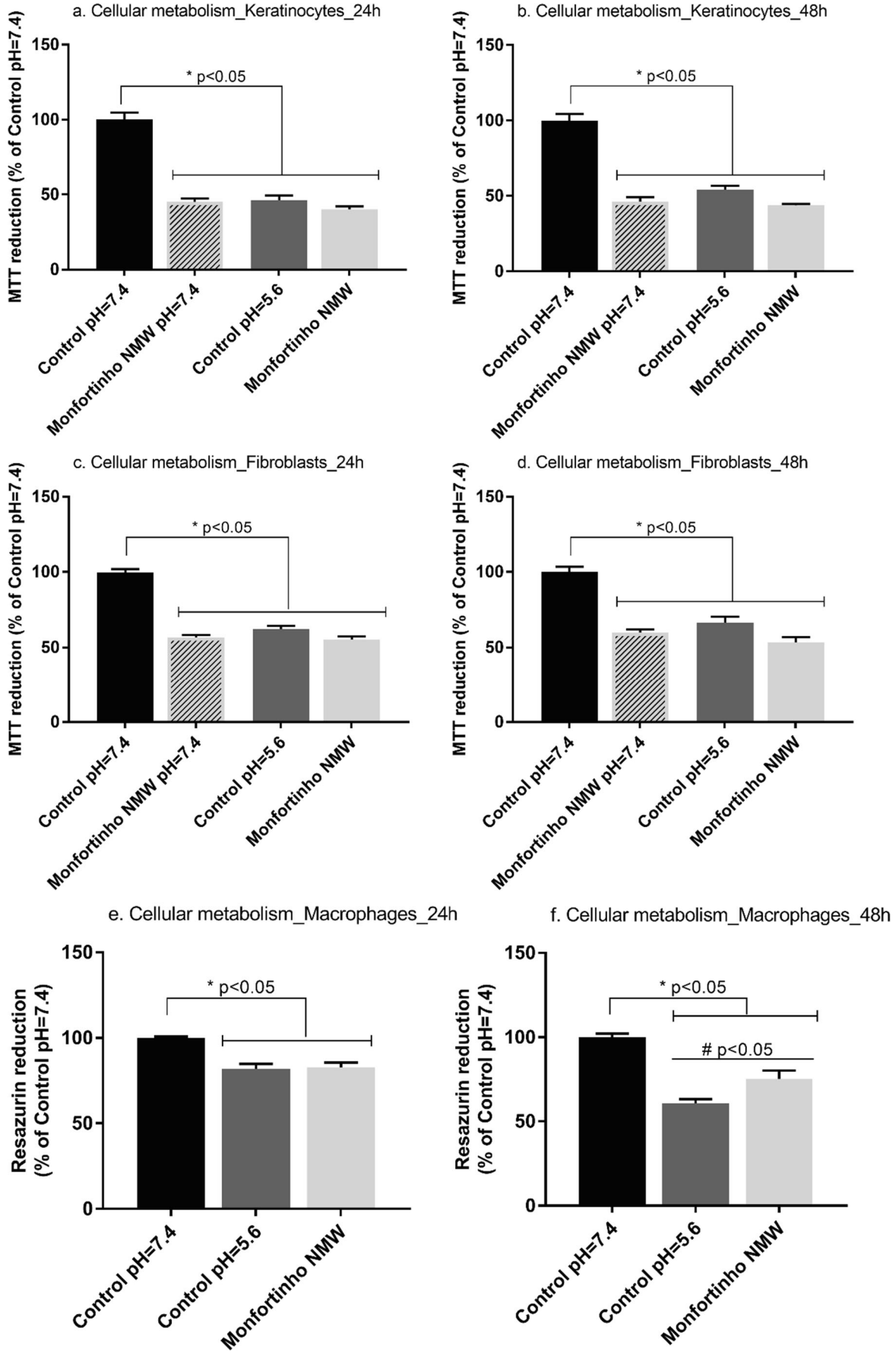
This significant reduction in cell proliferation appears not to be related to cell death as no significant increase in dead cells were counted in the trypan blue dye exclusion assay (data not shown).

Fibroblasts migration assay

The ability of NMW to influence fibroblasts motility was also evaluated (Fig. 3). When comparing the three tested conditions at $t = 0$ and $t = 12$ h, an overall increase on cell migration is visible. Monfortinho NMW induced a slight decrease in cell migration, compared to the control pH = 7.4, after 12 h of cell exposure. Although not so evident, a decrease in cell migration with culture medium at pH = 5.6 was also observed. However, no statistical significant differences were observed between the tested conditions (Fig. 3a).

Senescence-associated β-galactosidase in fibroblasts

To further investigate the effect of Monfortinho NMW on fibroblasts, the senescence state of cells after treatment with etoposide in the absence or presence of NMW was evaluated. As represented in Fig. 4, NMW significantly increased the number of etoposide-induced senescent cells when compared with the control group. This effect seems not to be dependenton the water's natural pH, since a significant difference was present between the number of senescent cells treated with NMW when compared with cells treated with control pH = 5.6.


a. Cellular metabolism_Keratinocytes_24h
MTT reduction (% of Control pH=7.4)
150
100
50
0
* p<0.05
Control pH=7.4
Monfortinho NMW pH=7.4
Control pH=5.6
Monfortinho NMW
b. Cellular metabolism_Keratinocytes_48h
MTT reduction (% of Control pH=7.4)
150
100
50
0
* p<0.05
Control pH=7.4
Monfortinho NMW pH=7.4
Control pH=5.6
Monfortinho NMW
c. Cellular metabolism_Fibroblasts_24h
MTT reduction (% of Control pH=7.4)
150
100
50
0
* p<0.05
Control pH=7.4
Monfortinho NMW pH=7.4
Control pH=5.6
Monfortinho NMW
d. Cellular metabolism_Fibroblasts_48h
MTT reduction (% of Control pH=7.4)
150
100
50
0
* p<0.05
Control pH=7.4
Monfortinho NMW pH=7.4
Control pH=5.6
Monfortinho NMW
e. Cellular metabolism_Macrophages_24h
Resazurin reduction (% of Control pH=7.4)
150
100
50
0
* p<0.05
Control pH=7.4
Control pH=5.6
Monfortinho NMW
f. Cellular metabolism_Macrophages_48h
Resazurin reduction (% of Control pH=7.4)
150
100
50
0
* p<0.05
# p<0.05
Control pH=7.4
Control pH=5.6
Monfortinho NMW

◀ **Fig. 1** Effect of NMW on cellular metabolic state. Keratinocytes, fibroblasts and macrophages were plated and exposed to Monfortinho NMW for 24 h (**a**, **c** and **e**, respectively) or 48 h (**b**, **d** and **f**, respectively). Alternatively, cells were exposed to culture medium with NMW pH (Control pH = 5.6) or to NMW at physiological pH (Monfortinho NMW pH = 7.4) to exclude the effect of the NMW intrinsic pH. MTT assay (keratinocytes and fibroblasts) or rezasurin assay (macrophages) were then performed to assess cell metabolic activity. Data correspond to the means ± SEM of three independent experiments and are represented as % of control prepared with MilliQ-type water at pH 7.4 - untreated cells (Control pH = 7.4, black bars). Statistical analysis: one-way ANOVA with Tukey's multiple comparison test; $*p < 0.05$ compared to control (Control pH = 7.4) was considered significant; $\#p < 0.05$ compared to pH control (Control pH = 5.6) was considered significant

Discussion

The mechanisms promoting the health benefits of NMWs are only partially understood and probably result from a combination of different factors, with mechanical, thermal and chemical effects among the most prominent ones (Sukenik et al. 1999; Fioravanti et al. 2011). While mechanical and thermal properties appear to act in a broad and unspecific manner, physicochemical properties seem to be water specific and may be partially related to skin absorption of organic substances or minerals that can act on a systemic level (Fioravanti et al. 2011). Additionally, even the relaxing and stress relief environment of a thermal centre can promote a general health improvement (Riyaz and Arakkal 2011).

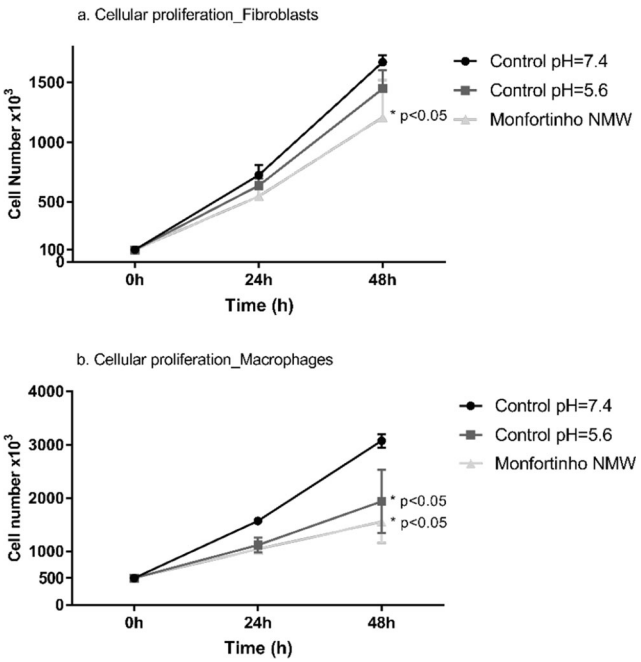


Fig. 2 Effect of NMW on cell proliferation. Fibroblasts (**a**) and macrophages (**b**) were plated and exposed to Monfortinho NMW for 24 h or 48 h. Cells were also exposed to culture medium with NMW pH (control pH = 5.6). The number of viable and dead cells was evaluated and cells were counted manually using a Neubauer chamber. Since the NMW had no effect on cell death (absence of dead cells), the results represent the total cell number counted. Data correspond to the mean ± SEM of three independent experiments and are represented as % control prepared with MilliQ-type water at pH 7.4 - untreated cells (Control pH = 7.4, black line). Statistical analysis: two-way ANOVA with Tukey's multiple comparison test; $*p < 0.05$ compared to control (Control pH = 7.4) was considered significant

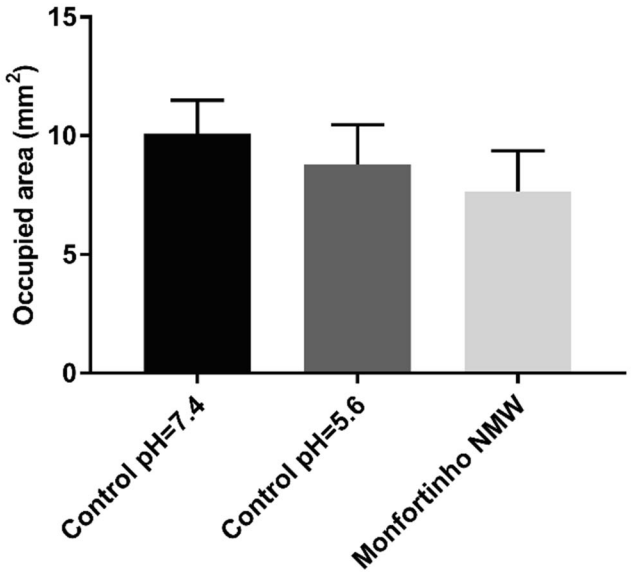
Therefore, the use of cell cultures can be useful in addressing the effects of NMW in a non-biased mode (Valitutti et al. 1990; Joly et al. 2000; Lee et al. 2012; Zöller et al. 2015a; Nicoletti et al. 2016). For this purpose, we made use of cell lines representative of different skin layers namely keratinocytes (epidermis) and skin fibroblasts (dermis) and macrophages (known to play a vital role in skin immunity) to study the effects of Monfortinho NMW in skin cellular response.

Regarding the NMW physicochemical properties, Monfortinho NMW is characterized by its acidic pH (5.6) which is in accordance with the "normal" pH of the skin surface of most body parts (pH 4.1 to pH 5.8). In inflammatory skin diseases, including AD and psoriasis, an increased pH is present, benefiting these conditions from the treatment with emollients with slightly acidic pH (Gubán et al. 2016). The fact that this NMW's pH is within the normal skin pH, can present an advantage in the treatment of these skin diseases, helping restoring the pH balance.

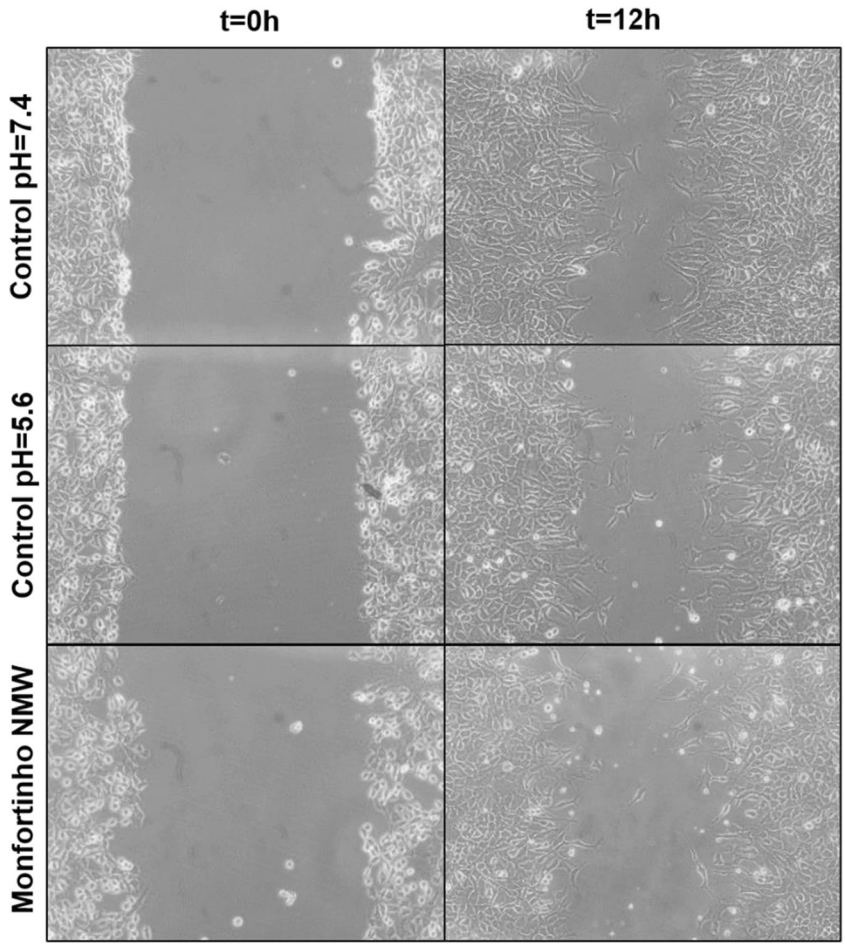
Another unique characteristic of this NMW is its high values of silica and silicate, as described in its physicochemical analytical sheet (Table 1). This high content in silica may account for its therapeutic indication in skin conditions, like psoriasis and AD, as silica is already described to have an abrasive effect in psoriatic plaques and an emollient effect when present in NMW (Coutinho et al. 2015). The beneficial effect of silica-rich NMWs was already addressed for other NMWs, like Avène thermal spring water, which has a high concentration of silica and calcium bicarbonate and was proved to reduce cutaneous basophil degranulation in AD and potentially prevented the itch-scratch cycle, prevalent in these patients (Joly et al. 2000).

Regarding the specific effects of silica-rich NMW in vitro, in our study, an overall reduction in metabolic activity was evident in three tested cell lines. Interestingly, no difference was present regarding the decrease in cell metabolism when exposed to NMW compared with MilliQ-type water at pH 5.6 (Control pH = 5.6). This led us to hypothesize that a non-specific, pH-dependent effect may account for this impairment in cell metabolism. However, when the intrinsic acidic pH of the NMW was altered to a physiological value of 7.4, this metabolic impairment was still present, which was unforeseen. A possible explanation is that the NMW has its own buffer effect, restoring its natural pH (in this case acidic pH) as denoted by visual inspection of the culture medium. This buffering effect is reported on the literature regarding both NMW and cosmetic products that have NMW in their composition

a. Cellular migration_Fibroblasts_12h



b. Representative images of the effect of Monfortinho NMW on the migration of fibroblasts



◀ **Fig. 3** Graphical representation (**a**) and representative images (**b**) of the effect of NMW on the migration of fibroblasts. Cells were plated and allowed to achieve 95% confluence. The scratch assay was performed as described in "Material and methods." The images were acquired by inverted microscopy coupled with a digital camera. Photographs were taken before cell exposure ($t = 0$ h) and 12 h after exposure to Monfortinho NMW and controls. Magnification used was ×100 (10 × 10). Data represented in the graphic correspond to the mean ± SEM of three independent experiments and are represented as % of control prepared with MilliQ-type water at pH 7.4 - untreated cells (Control pH = 7.4, black bars). Statistical analysis: one-way ANOVA with Tukey's multiple comparison test; $*p < 0.05$ compared to Control pH = 7.4 was considered significant. Representative examples of decreased migration capacity of fibroblasts when exposed to NMW are presented in **b**

(Proksch 2018). On the other hand, other intrinsic characteristics besides pH, such as specific ions or osmolality values, can contribute to this decrease in cell metabolism, as reported by other authors for different NMWs (Lee et al. 2012; Zöller et al. 2015b). Nadja Zöller et al. showed that La Roche Posay and Avène NMWs significantly decreased basic parameters such as keratinocyte proliferation, and hypothesized that trace elements such as selenium and zinc were critical for this effect (Zöller et al. 2015b). Also Ho-Pyo Lee and his team, observed a similar decrease in the viability of a keratinocyte cell line when tested with undiluted spring water and this reduction seemed to be related with the water's high osmolality, since decreasing water's concentration led to a restoration of cell metabolism (Lee et al. 2012). This diversity of contributing factors, rather than a specific attribute or chemical element, may account for a wide spectrum 'whole-water effect'. The conclusion that a specific effect can be due to the water as a whole (or a combination of elements) rather than to a particular chemical element has already been drawn by different authors (Araujo et al. 2017; Varga 2019; Cheleschi et al. 2020).

In addition to skin keratinocytes, also skin fibroblasts had their metabolism and proliferation reduced when exposed to Monfortinho NMW. One possible explanation for impairment of cell metabolism and proliferation in fibroblasts is cellular senescence. Senescent cells are characterized by their inability

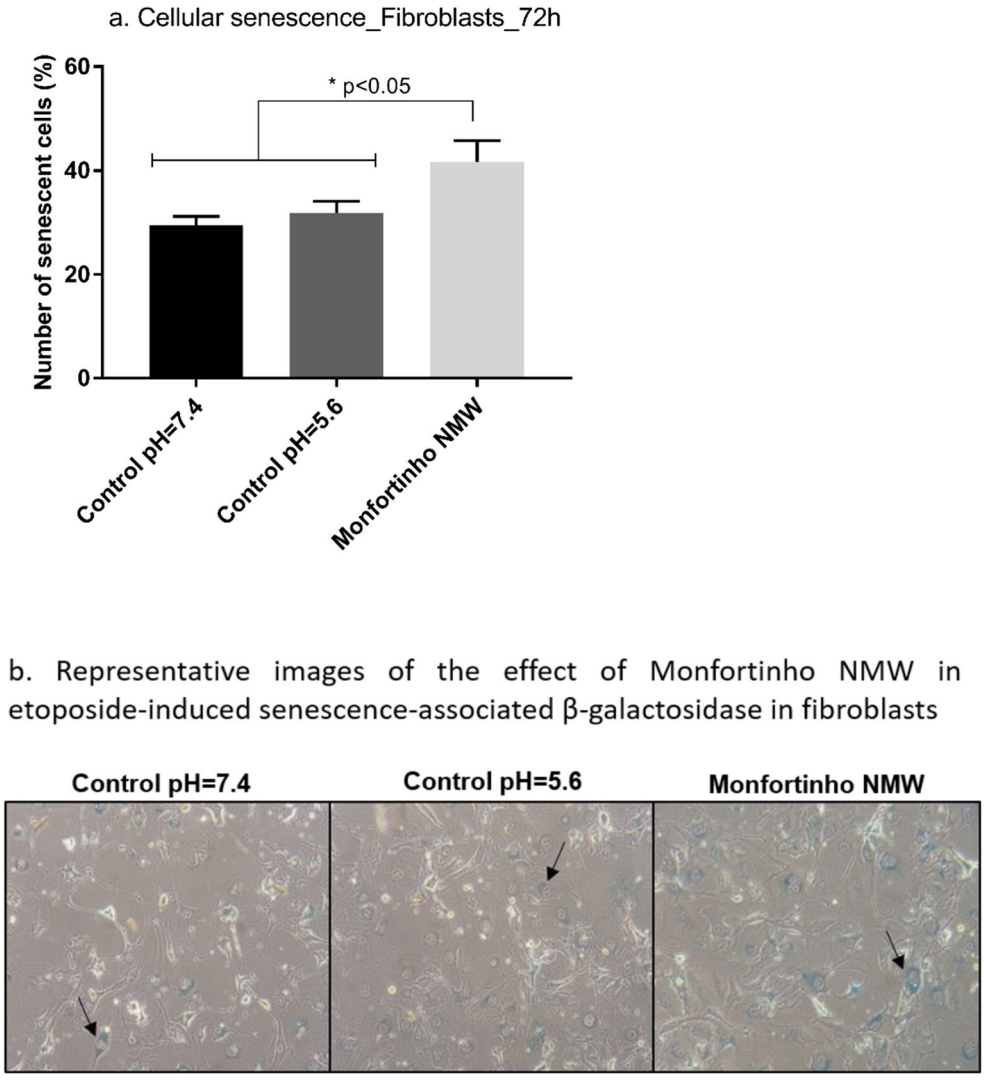


Fig. 4 Graphical representation (**a**) and representative images (**b**) of the effect of NMW in etoposide-induced senescence-associated β-galactosidase in fibroblasts. Fibroblasts were plated and allowed to adhere overnight. Senescence was induced with 12.5 μM of etoposide for 24 h. After 72 h of cell recovery in Monfortinho NMW and different controls, percentage of senescent associated β-galactosidase positive cells was quantified in different groups by cell counting. Statistical analysis: one-way ANOVA with Tukey's multiple comparison test; $*p < 0.05$ compared to Control pH = 7.4 was considered significant. Representative examples of increased senescence-associated β-galactosidase (blue stained cells) is presented in **b**. Magnification used was ×100 (10 × 10)

to proliferate, resistance to apoptosis and secretion of factors that promote inflammation and tissue deterioration (Wang and Dreesen 2018). These cells display an enlarged and flattened cell shape and elevated senescence-associated β-galactosidase activity, which remains the gold standard to identify senescent cells in culture and tissue samples (Wang and Dreesen 2018). As presented in Fig. 4, cell treatment with NMW enhanced the number of senescent cells.

The abovementioned inhibitory impact in cellular proliferation and metabolism caused by exposure to silica-rich NMW appears to be water specific, as other NMW studied by different authors appear to cause opposite effects, increasing cell migration and proliferation (Faga et al. 2012; Nicoletti et al. 2017). Faga et al. used an Italian calcium magnesium bicarbonate-based spring water (Comano, Italy) in an in vivo rabbit wound model and identified that the topical administration of the Comano spring water increased keratinocyte proliferation and migration (Faga et al. 2012). Additionally, Nicolleti et al. used the same Comano water in cultured human skin fibroblasts and observed that cells exposed to 20% Comano NMW exhibited a 31% higher proliferation value than controls maintained in conventional culture media (Nicoletti et al. 2017). This leads us to conclude that different NMWs produce different effects, thus justifying the different claims and empirical uses of each water and making each specific NMW valuable assets for different skin applications.

Finally, the exposure of macrophages to Monfortinho NMW produced a moderate, but still significant, decrease in cell metabolism. In addition, Monfortinho NMW produced a remarkable decrease in cell proliferation in these cells.

From a pathophysiological perspective, psoriasis is largely caused by an imbalance between the local immune response and its regulatory mechanisms (Arasa et al. 2019) Abnormal keratinocyte proliferation and epidermal turnover time are the main pathological features of psoriasis (Nedoszytko et al. 2014; Li et al. 2015). Also, the epithelial cells of the skin are hyper-proliferative and fail to undergo normal differentiation, leading to a marked thickening of the epidermis (Leite Dantas et al. 2016). Based on this information, the effect of NMW in reducing metabolic activity of keratinocytes may support the existing empirical and clinical evidenced success in symptom relief in psoriatic patients.

Additionally, a well-established feature of psoriasis is the dense infiltration of cells of the innate immune system, namely macrophages, especially around the epidermal/dermal interface (Leite Dantas et al. 2016). This mechanism appears to be site specific in psoriatic patients as macrophages accumulate only in the damaged skin area when compared with adjacent non-injured skin in the same patients (Leite Dantas et al. 2016). Therefore, a decrease in cell metabolism of macrophages after exposure to NMW and, most importantly, a remarked reduction in cell proliferation may also be involved in the beneficial effect of this NMW in patients with psoriatic lesions.

Regarding fibroblasts, the role of these cells in psoriasis is yet to be clarified (Arasa et al. 2019). There is evidence suggesting that fibroblasts from skin of patients with psoriasis induce keratinocyte outgrowth (Gubán et al. 2016). In these patients, the fibroblast-keratinocyte crosstalk is responsible for the hyper-proliferation of keratinocytes (Berroth et al. 2013). Therefore, reduced metabolism and proliferation of fibroblasts during specific skin conditions, such as psoriasis, may be beneficial. Less responsive or senescent fibroblasts would not induce hyper-proliferation of keratinocytes, which might be beneficial in the case of psoriatic lesions.

The use of NMW for the relief of skin conditions is well established. Although these beneficial effects justify the high demand for balneotherapy to alleviate inflammatory skin conditions, as psoriasis and AD, scientific support as a differentiating factor of Portuguese balneotherapy to sustain its use is of utmost importance, similar to what happens in several other European countries. Indeed, independent scientific investigations are rare and necessary to provide a more solid basis to evaluate spa water-mediated effects (Zöller et al. 2015b).

To the best of our knowledge, our study provides the first insights on the effect of Monfortinho NMW on the metabolism and proliferation of skin and inflammatory cells.

Since skin conditions that benefit from the use of this particular NMW are characterized by a hyper-proliferative state of epidermal skin cells and an increased infiltration of inflammatory cells, the reduction on cell metabolism and proliferation of these cells may play a role on some beneficial effects found in vivo.

Still, it is important to emphasize that the present study represents a preliminary investigation of the cellular effects of a silica-rich NMW and results here presented intend to scientifically sustain some of the beneficial empirical effects on skin conditions attributable to this NMW without scrutinizing the associated molecular pathways involved, which was beyond the scope of this study. Additionally, since only in vitro studies were preformed, some intrinsic characteristics recently considered to be responsible for some positive effects of NMWs in different skin diseases, as water's microbiome (composed of non-pathogenic, autochthonous organisms), cannot be taken into account due to the filtration procedures applied (Varga 2019; Cheleschi et al. 2020).

Overall, our results contributed to the scientific validation of the beneficial effects of one of the oldest Portuguese silica-rich NMWs, by addressing fundamental mechanisms of skin cell homeostasis, known to be impaired on hyperkeratotic skin conditions. In the future, further studies focused on the molecular and cellular mechanisms evoked by this NMW, using complex cellular models, as 3D models, primary cultures or tissue explants derived from diseased skin, should be pursued

in order to specifically investigate the NMWs' beneficial effect in particular skin diseases.

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Authors' contributions ASO performed the experiments, assembled and analysed the data, and wrote the manuscript. CVV and AS collaborated in the acquisition and assembly of data and critically revised the manuscript. SC, RF, LB, JMO, RPO and CP contributed to experimental conception and design, and critical reading and editing of the manuscript. MTC and APO contributed to experimental conception and design, data analysis and interpretation and revised the manuscript critically for important intellectual content.

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Data Availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics approval This article does not contain studies with human participants or animals performed by any of the authors.

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