

Impact of Different Spa Waters on Inflammation Parameters in Human Keratinocyte HaCaT Cells

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Background: The treatment of different skin conditions with spa waters is a long tradition dating back to at least late Hellenism. Interestingly, independent scientific examinations studying the effect of spa waters are scarce. **Objective:** In the present in vitro study, we compared the effect of culture media supplemented with (a) thermal spa waters (La Roche-Posay, Avène) and (b) two natural mineral drinking waters (Heppinger, Adelholzener) on physiological parameters in HaCaT keratinocytes. **Methods:** The different medium preparations were investigated with regard to cell proliferation and cell damage. Moreover, the impact on inflammation parameters with and without ultraviolet B (UVB) irradiation was examined. **Results:** Two popular thermal spring waters were found to suppress cell proliferation and cell damage. Moreover, these waters reversed the induction of interleukin-6, as measured using enzyme-linked immunosorbent assay and promoter transactivation, and the formation of reactive oxygen species after UVB stimulation. Of note, the two natural mineral waters, which are distributed as drinking waters, had some effect on the above-mentioned parameters but to a lesser extent. **Conclusion:** In summary, our results show that spa waters, and particularly those de-

rived from thermal springs, reduce parameters associated with inflammation. It seems likely that trace elements such as selenium and zinc are critical for the observed effects. (**Ann Dermatol 27(6) 709 ~ 714, 2015**)

-Keywords-

Inflammation, Interleukin-6, Keratinocytes, Reactive oxygen species, Spa water, Thermal spring water

INTRODUCTION

In ancient times, when therapeutic options were much more limited than they are today, the use of systemic and external spa waters for the treatment of different physiological conditions was very popular. Even now, countless health resorts with specific spa waters continue this tradition. In dermatology, balneotherapy is mainly used for the treatment of psoriasis and atopic dermatitis. In particular, the water from the Dead Sea in combination with UV light is reported to alleviate the symptoms of psoriasis¹. Spa waters from other sources are also reported to be useful in the treatment of inflammatory skin diseases²⁻⁶. However, it is difficult to attribute the measured effects to specific parameters. There are data suggesting that the chemical and thermal properties of spa water have an impact on skin cell physiology². Moreover, the mechanical effects of application (immersion, hydromassage, high-pressure showers) trigger physiological changes in the renal and cardiovascular systems with potentially beneficial effects on skin diseases⁷. Finally, the relaxing environment of a health spa can also aid in general health improvement with impacts on the skin.

The use of cell cultures is useful for determining the effects of spa water more precisely. The present study was

Received January 20, 2015, Revised February 5, 2015, Accepted for publication March 5, 2015

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aimed at testing two popular thermal spa waters from La Roche-Posay (LRP; L'Oréal, Clichy, France) and Avène (ASW; Pierre Fabre, Paris, France) in direct comparison in a keratinocyte model (HaCaT). For this study, HaCaT cells were chosen, as they have been shown to respond similar to primary skin keratinocytes in previous assays^{8,9}, but as a cell line, they lack interindividual variability. In this regard, proliferation, cytotoxicity, interleukin-6 (IL-6) expression, and formation of reactive oxygen species (ROS) after stimulation with ultraviolet B (UVB) were tested. Besides regular culture conditions, using double-distilled mineral-free water as a control, two natural mineral drinking waters (Adelholzener [St. Primus Heilwasser, Bad Adelholzen, Germany], Heppinger [Apollinaris, Bad Neuenahr-Ahrweiler, Germany]) were also included in the test.

MATERIALS AND METHODS

Cell culture

The spontaneously immortalized human keratinocyte cell line (HaCaT) (a generous gift from Norbert Fusenig, German Cancer Research Institute, Heidelberg, Germany) was cultured in carbonate-buffered Hank's medium with 5% fetal calf serum and 1% penicillin/streptomycin solution (Biochrom KG, Berlin, Germany) at 37°C in a 5% CO₂ atmos-

phere. Medium components in powdered form were solubilized using sterile double-distilled water (control) or spa water, for a concentration of 72% in the final medium. For spa water supplementation, (a) two thermal spring waters from LRP (L'Oréal) and ASW (Pierre Fabre) and (b) two natural mineral waters distributed as drinking waters from Heppinger and Adelholzener were used. The composition of the spa waters is given in Table 1. All experiments were performed in agreement with the local ethics commission.

DNA synthesis

HaCaT cells were cultivated in microwell plates at a density of 2×10^4 cells/0.33 cm². Cells were exposed for 24 h to spa water-supplemented medium. For the last 16 h, cells were pulsed with 5-bromo-2'-deoxyuridine (BrdU). Subsequently, the incorporation rate of BrdU was determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Roche, Mannheim, Germany). Briefly, cells were fixed and immune complexes were formed using peroxidase-coupled BrdU-antibodies. A colorimetric reaction with tetramethylbenzidine (TMB) as a substrate gave rise to a reaction product measured at 450 nm in a scanning multiwell spectrophotometer (ELISA reader, MR 5000; Dynatech, Guernsey, UK).

Table 1. Composition of spa waters as provided by the manufacturers¹⁴

Concentration	Heppinger extra	Adelholzener St. Primus Heilwasser	La Roche-Posay	Avène
Silica (mg/L)	41.7	10.2	31.6	14
Bicarbonates (mg/L)	2,495	412	387	226.7
Sulfate (mg/L)	60	8	ND	13.1
Chloride (mg/L)	118	2.8	ND	5.4
Nitrate (mg/L)	ND	3.4	ND	1.4
Fluoride (mg/L)	0.52	0.07	ND	0.1
Phosphate (mg/L)	0.06	ND	ND	0.3
Calcium (mg/L)	150	88	149	42.7
Magnesium (mg/L)	199	29	4.4	21.2
Potassium (mg/L)	27.1	0.6	ND	0.8
Sodium (mg/L)	481	3.7	ND	4.8
Iron (mg/L)	0.009	ND	ND	<0.1
Manganese (mg/L)	0.007	ND	ND	<0.1
Strontium (mg/L)	ND	0.14	ND	0.1
Lithium (mg/L)	0.84	ND	ND	<0.1
Boron (μ g/L)	5,720	230	ND	220
Zinc (μ g/L)	ND	ND	<5	20
Cadmium (μ g/L)	ND	ND	ND	2
Copper (μ g/L)	ND	ND	ND	<5
Selenium (μ g/L)	ND	ND	53	<5
Barium (μ g/L)	ND	56	ND	220
Carbon dioxide (mg/L)	2,760	2,160	ND	ND

ND: not determined.

Membrane integrity

Cell lysis was quantified using the cytotoxicity detection kit (Roche), which is based on the release of lactate dehydrogenase (LDH) from damaged cells. Briefly, HaCaT cells were seeded in microwell plates as described above and were treated with spa water-supplemented medium for 24 h. As a positive control (maximal cell damage), cells were treated with 1% Triton X-100 (Merck, Darmstadt, Germany). Subsequently, the cell-free supernatants were incubated with NAD⁺, which becomes reduced by LDH to NADH/H⁺. In a second step, NADH/H⁺ reduces a yellow tetrazolium salt to a red-colored formazan salt. The amount of red color is proportional to the number of lysed cells. For quantitation, the absorbance of the reaction product was measured at 490 nm using a multiwell spectrophotometer.

Enzyme-linked immunosorbent assay of IL-6

After preincubation for 1 h in the presence or absence of spa water-supplemented medium, HaCaT cells in phosphate-buffered saline, also reconstituted with the spa waters or double-distilled water, were irradiated with 150 mJ/cm² UVB using a Psorilux-UVB-lamp (Heraeus, Hanau, Germany). Thereafter, cells were incubated with or without spa water-supplemented medium for 24 h. Then, cell-free

supernatants were obtained and assayed for human IL-6 using commercial ELISA test kits (R&D Systems, Wiesbaden, Germany). Betamethasone-17-valerate (10 µg/ml) served as a positive control; dimethyl sulfoxide (DMSO) served as a solvent control. Briefly, supernatants were placed in microwell plates coated with antibodies against IL-6. After incubation with a horseradish-peroxidase conjugate, TMB (Sigma-Aldrich, Steinheim, Germany) was added, giving rise to a colored product measured at 450 nm in a scanning multiwell spectrophotometer (ELISA reader MR 5000).

IL-6 promoter transactivation assay

The human IL-6 promoter construct spanning 1,168 bp linked to a luciferase reporter gene in pGL3 basic (Promega, Mannheim, Germany) was a kind gift from Michèle Resche-Rignon¹⁰. Constructs were transfected into subconfluent HaCaT cells by lipofection (Lipofectamine reagent 2000; Invitrogen, Darmstadt, Germany). In order to standardize transfection efficacy, cells were co-transfected with a humanized *Renilla* luciferase vector (pRL; Promega). Transfected cells were treated with spa water-supplemented medium, betamethasone-17-valerate as positive control, or DMSO as a solvent control for 24 h. After irradiation with 150 mJ/cm² UVB, cells were propagated for an additional 24 h in the above-mentioned media. Then, cells were lysed and activities of both luciferases were de-

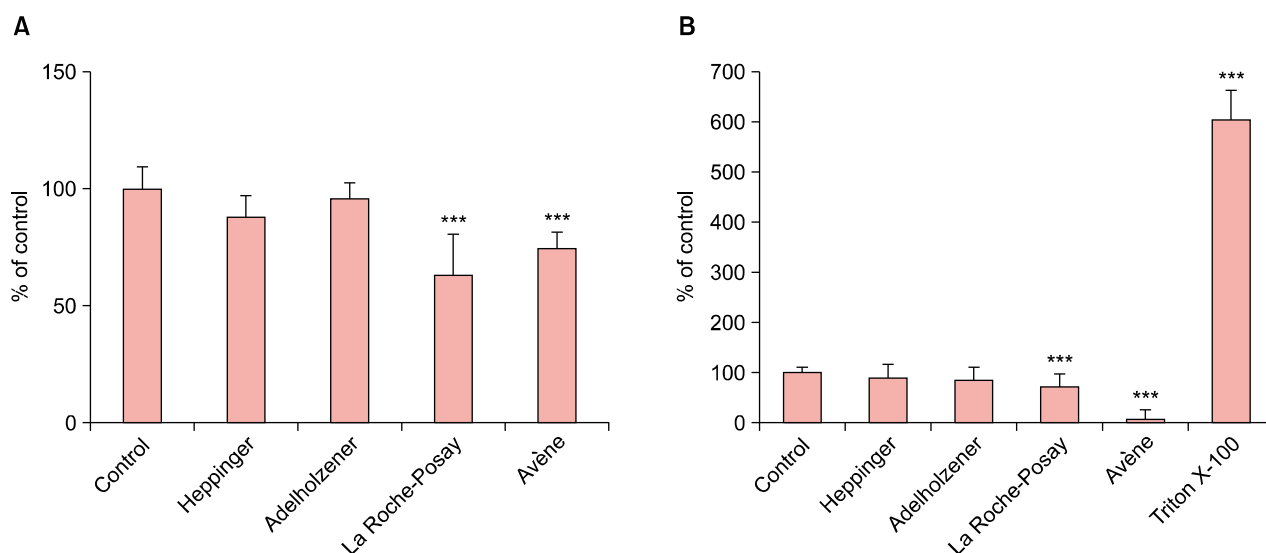


Fig. 1. Effect of spa water-supplemented medium on DNA synthesis and membrane integrity. Human skin keratinocytes (HaCaT) were cultured in regular medium (control) or in medium supplemented with 72% spa water from different sources. After 24 h, (A) the incorporation of 5-bromo-2'-deoxyuridine (BrdU) in the DNA and (B) the amount of lactate dehydrogenase (LDH) in supernatants were detected as described in "Materials and Methods." The complete release of LDH was achieved by treatment with 1% Triton X-100. Each bar represents the mean of 18 (A) and 24 (B) independent experiments. Standard deviations are indicated. Data were compared to untreated controls. Heppinger: Apollinaris, Bad Neuenahr-Ahrweiler, Germany. Adelholzener: St. Primus Heilwasser, Bad Adelholzen, Germany. La Roche-Posay: L'Oréal, Clichy, France. Avène: Pierre Fabre, Paris, France. Triton X-100: Merck, Darmstadt, Germany.

tected separately, using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Berthold, Bad Wildbad, Germany).

Measurement of reactive oxygen species

HaCaT cells were incubated with spa water-supplemented

medium in the presence of 100 μ M 1,2,3-dihydrorhodamine (DHR 123; Sigma-Aldrich) or with 100 or 200 μ M vitamin C, a known radical scavenger, as a positive control for 1 h at 37°C. Subsequently, the medium was substituted with PBS and cells were irradiated with 400 mJ/cm² UVB. After incubation for 45 min, the fluorescence

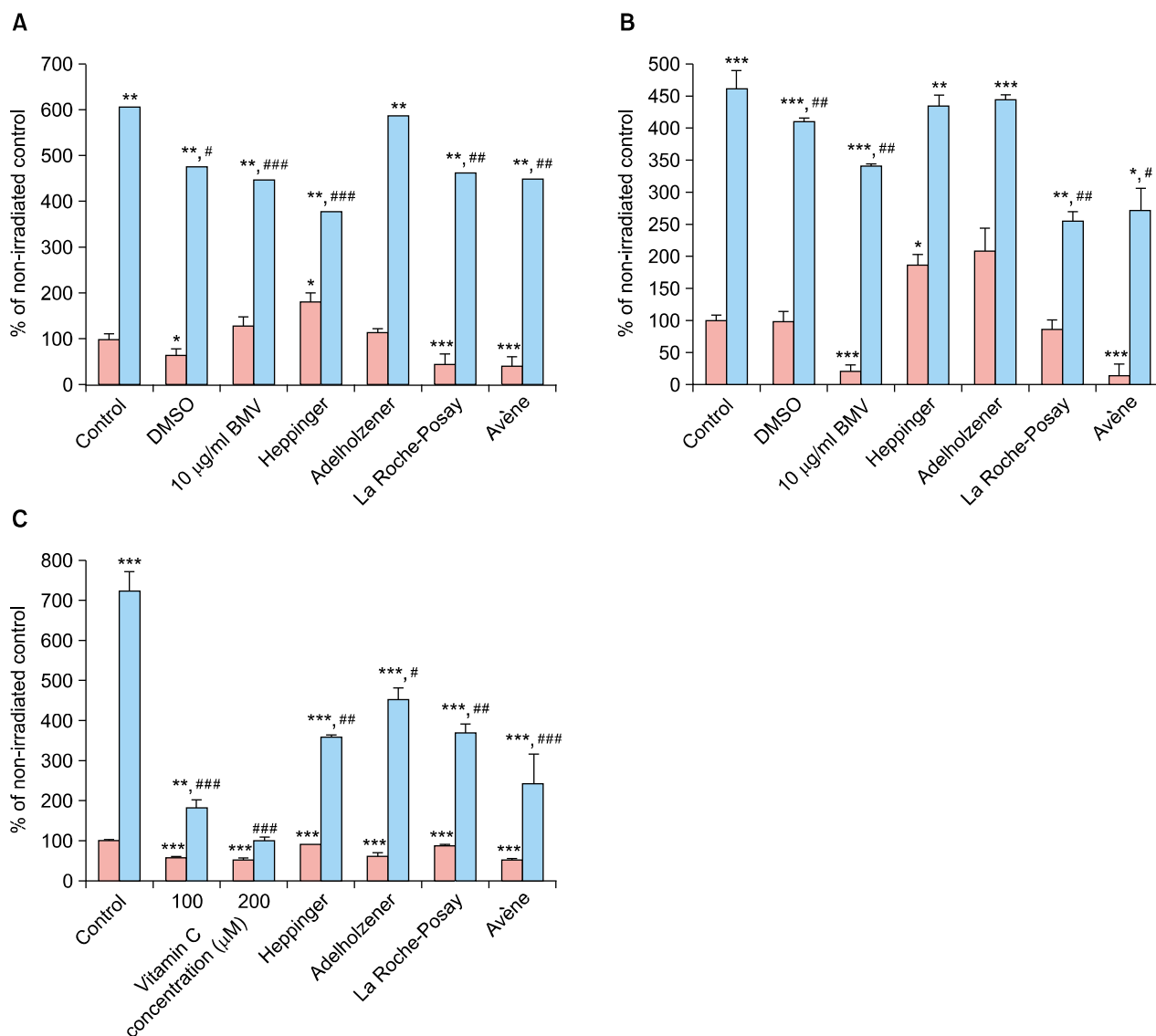


Fig. 2. Effect of spa water supplementation on inflammation parameters in human keratinocytes. (A) Irradiated (black bars) and non-irradiated (white bars) cells were held for 24 h in medium supplemented with 72% spa waters as described. In cell-free supernatants, the amount of interleukin-6 (IL-6) was determined by an enzyme-linked immunosorbent assay. A treatment with betamethasone-17-valerate served as a positive control. (B) Cells transfected with an IL-6 promoter-based luciferase construct were irradiated or non-irradiated. After 24 h, luciferase activity was measured. Transfection efficacy was controlled by co-transfection with a humanized *Renilla* luciferase vector (phRL; Promega, Mannheim, Germany). (C) The formation of reactive oxygen species was measured in DHR 123 (Sigma-Aldrich, Steinheim, Germany)-loaded cells treated with UVB, as described above. Vitamin C served as a positive control. Each bar represents the mean of 4 (A), 4 (B), and 8 (C) independent experiments. Standard deviations are indicated. Data were compared to that for untreated (asterisks) or irradiated (hash signs) controls. DMSO: dimethyl sulfoxide, BMV: betamethasone-17-valerate. Heppinger: Apollinaris, Bad Neuenahr-Ahrweiler, Germany. Adelholzener: St. Primus Heilwasser, Bad Adelholzen, Germany. La Roche-Posay: L'Oréal, Clichy, France. Avène: Pierre Fabre, Paris, France.

was quantitatively detected using a multiwell spectrofluorometer (Cytofluor; Applied Biosystems, Langen, Germany) equipped with 485 nm excitation and 560 nm emission filters.

Presentation of data and statistical analysis

All data are presented as mean values \pm standard deviations. Statistical significance in the data was evaluated by t-test (BIAS, Frankfurt, Germany). Each set of data relates to the untreated (asterisk) or irradiated (hash) control as indicated. Differences were considered significant at a level of $p < 0.05$, represented by one icon, $p < 0.01$, represented by two icons, and $p < 0.001$, represented by three icons.

RESULTS AND DISCUSSION

A general problem of studies dealing with spa waters is that most of these studies are initiated by the spa water manufacturers themselves. Independent scientific investigations are rare and necessary to providing a more solid basis to evaluate spa water-mediated effects. Here, we show that basic parameters such as proliferation and cytotoxicity were significantly decreased by LRP and ASW (Fig. 1). Moreover, both thermal waters reduced IL-6 levels in the medium after UVB irradiation to levels similar to that seen with betamethasone-17-valerate treatment (Fig. 2A). Interestingly, the drinking water from Heppinger also induced strong downregulation of IL-6. The IL-6 levels measured from non-irradiated cells were all at the detection limit of the test, making reliable statements infeasible. In the transactivation assay, both thermal waters showed a clear inhibitory effect on IL-6 after UVB stimulation, suggesting regulation at the promoter level (Fig. 2B). Finally, the formation of ROS after UVB was attenuated by LRP and ASW. Of note, both drinking waters from Heppinger and Adelholzener also showed distinct ROS suppressions (Fig. 2C).

Particularly in the last few decades, the French cosmetic industry has marketed thermal spa waters as cosmeceuticals, requiring some effort to prove cellular effects. The regulation of immunomodulatory parameters by spa water-supplemented media was observed in mast cells (ASW)¹¹, Langerhans cells (LRP)¹² and CD4⁺ T lymphocytes (Yong-gung oncheon, ASW)^{13,14}. In the latter, a partial shift from a Th2 to a Th1 cytokine profile was observed, offering a rationale for the treatment of atopic dermatitis¹⁴. Preliminary studies using cultured fibroblasts suggest enhanced plasma membrane fluidity by ASW¹⁵. Moreover, differentiation of skin keratinocytes as measured by the expression of involucrin and cytokeratins 1 and 10 was induced by ASW¹⁶.

Our results show that, although the thermal waters LRP and ASW differed in composition, they both efficiently suppressed the induction of a prototypical inflammatory cytokine and the formation of ROS after UVB. It seems likely that, in the case of LRP, the observed effects are mediated by the high selenium content (53 $\mu\text{g/L}$), which is a co-factor for glutathione peroxidase, a key-enzyme in the elimination of ROS¹⁷. Interestingly, ASW, which is almost free of selenium ($< 4 \mu\text{g/L}$) showed anti-inflammatory properties. Here, the relatively high zinc content (20 $\mu\text{g/L}$) seems responsible for anti-inflammation¹⁸. Unfortunately, the composition list for the two mineral waters, as provided by the manufacturers, is incomplete. However, it could be speculated that boron, a trace element shown to act on keratinocytes¹⁹, is involved in the observed effects. Further studies should address these hypotheses.

In summary, our results give a scientific rationale for the application of spa waters in the treatment of chronic inflammatory skin diseases.

ACKNOWLEDGMENT

The study was supported by Prof. Harry und Rosa Neumann-Stiftung and Dr. Paul and Cilli Weil Stiftung.

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