Vichy Thermal Spring Water (VTSW), a cosmetic ingredient of potential interest in the frame of skin ageing exposome: an in vitro study

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Abstract

OBJECTIVE: To study the effects of the very high minerality Vichy Thermal Spring Water (VTSW) on human keratinocytes grown in vitro.

METHODS: The effect of VTSW was monitored by full genome transcriptomic technology and immunofluorescence microscopy.

RESULTS: In the presence of 50% VTSW, the expression of a number of skin homoeostasis-related genes is increased, specifically with respect to dermal-epidermal junction, epidermal cohesion and communication, keratinocyte proliferation–differentiation balance, antioxidant mechanisms and DNA repair.

CONCLUSION: This work suggests that VTSW could be considered as an ingredient of potential interest to address some of the deleterious effects of skin ageing exposome.

Introduction

Skin ageing results from the cumulative effects of chronological ageing and environmental factors and is clinically and instrumentally characterized by skin dullness, dryness, laxity and wrinkles formation. From a histological point of view, skin ageing is mainly characterized by dermal-epidermal junction (DEJ) flattening, basement membrane delamination and alteration of the superficial dermis, with a strong decrease in type I collagen, glycosaminoglycan and proteoglycan content [1–4], type VII collagen [5], integrin β4 and laminin 312 [6]. It also results in an altered balance between keratinocyte proliferation and differentiation, resulting in barrier function disruption, reduction in structural integrity and loss of physiological function [7]. Both intrinsic and extrinsic ageing processes involve a number of factors, such as ROS (reactive oxygen species) and inflammatory mediators [8, 9]. As a matter of fact, human skin is daily exposed to free radicals due to endogenous processes linked, for example, to mitochondrial oxidative phosphorylation, and environmental insults, particularly solar radiation. Even though oxidative stress is important for physiological responses, sustained ROS production can end up with cell damage and lead to cells’ dysfunction [10]. Indeed, oxidative stress promotes tissue inflammation through upregulation of genes that encode for pro-inflammatory cytokines and sustained activation of NF-κB signalling pathway, highly suspected to accelerate skin ageing process [11–14] and more specifically inflam-m-ageing through activation of the innate immunity system [15]. This low-grade, continuous (chronic) inflammation and upregulation of pro-inflammatory mediators (IL-6, IL-8, TNFα…) are referred to as skin inflam-m-ageing [16]. In addition to UV rays [17], the increase in air pollution over the years has been suspected to impact human skin [18, 19]. For example, cell membrane can be damaged from lipid oxidation induced by free radicals and cigarette smoke [20]. Strikingly, two recent clinical studies performed in a polluted environment showed significant differences in superficial biochemical skin parameters measured on the face of volunteers [21,22], squalene peroxides appearing as a new and reliable marker of environmental pollution [23]. Mirroring the cutaneous responses to environmental stress [24], the major environmental factors that influence skin ageing have been recently regrouped in the so-called skin ageing exposome which includes (i) sun radiations, that is ultraviolet, visible light and infrared wavelengths, (ii) air pollution, (iii) tobacco smoke, (iv) nutrition, and (v) other factors such as temperature, stress and lack of sleep [18] that can alter skin conditions but still remain to be further studied.

Considering the age- and exposome-related alterations of skin structure and functions, one might consider that various cations such as calcium (Ca2+) is a key factor that controls keratinocyte terminal differentiation and more specifically integrin conformation, cadherin activity, desmosome formation and...
Manganese (Mn³⁺) is essential for mitochondrial superoxide dismutase (SOD) activity, whereas magnesium (Mg²⁺) is a key regulator of cell respiration and phosphorylation [27]. K⁺, through specific channels, mediates Ca²⁺-induced keratinocyte differentiation [28], whereas sodium (Na⁺) in aqueous solutions has water-binding properties and can be considered as a skin moisturizer [29].

Of note, Vichy Thermal Spring Water (VTSW) is characterized by a very specific ionic composition (Table I). It was previously found to stimulate the activity of Stratum corneum Catalase, an antioxidant enzyme that degrades hydrogen peroxide (H₂O₂) into water and oxygen, H₂O₂ being a potent initiator of ROS [30]. To further characterize the effects of VTSW on skin cells, normal human keratinocytes were grown in vitro in the presence or absence of VTSW, and a full genome gene expression profile was established. In parallel, the effect of VTSW on the in vitro expression of keratinocyte late differentiation markers was studied by immunofluorescence, in comparison with Ca²⁺.

Materials and methods

Keratinocyte Culture and treatment with Vichy Thermal Spring Water

To avoid possible bias due to single donor intrinsic variability, pooled foreskin Normal Human Epidermal Keratinocytes (NHEK) were obtained from Lonza (Amboise, France, CC-2507) and cultured in MCDB153 reconstituted growth medium (US Biological®, Salem, MA, USA, powder K0200) solubilized either in normal water or in water containing 50% of VTSW, supplemented with Human Keratinocyte Growth Supplement (HKGS, S-001-5, Invitrogen/Thermo Fisher Scientific, Villebon-sur-Yvette, France), gentamicin (Invitrogen, 15710-049), and 0.03 mM calcium chloride (CaCl₂). Keratinocytes were grown for 18 h before RNA extraction. These culture conditions were treated in triplicate.

RNA extraction

The samples were rinsed in Dulbecco’s Phosphate Buffer Saline (PBS). Gibco BRL/Thermo Fisher Scientific, Villebon-sur-Yvette, France). The total RNA was obtained according to the manufacturer's instructions using Rneasy mini-kit (Qiagen, Courtaboeuf, France). The amount of total RNA was quantified with Ultraspec 1100 Pro (Amersham, Saclay, France). The quality and integrity of RNA were analysed using a 2100 Bioanalyzer (Agilent, Courtaboeuf, France).

Microarrays hybridization and data acquisition

For each sample, 50 ng of RNA was treated with the Ovation Pico WTA System V2 (NuGEN, 3302-12, NuGEN, Leeks, The Netherlands). Then, 5 μg was fragmented and labelled with biotin (NuGEN Encore Biotin Module, 4200-12) and hybridized on Human Gene 2.0 ST DNA chips (Affymetrix/Thermo Fisher Scientific, Villebon-sur-Yvette, France, Affymetrix, 902112). The hybridization, washing and fixing were carried out according to Affymetrix protocol. Revelation step was performed with GeneChip Fluidics Station 450 (Affymetrix, 60-0079) and signal measurement with the GeneChip Scanner 3000 (Affymetrix).

Microarrays statistical analyses and selection of differentially expressed genes

Treatment of raw signals was performed through the ‘R’ software (v3.1.1) [31] and ‘oligo’ package (v1.30.0) from BioConductor project (v3.0) [32]. Data normalization was carried out through the RMA algorithm [33,34]. The statistical analysis was performed gene by gene and significantly differentially expressed genes between treated and untreated samples were identified using contrasts based on moderated t-statistics, performed with the R package ‘Llimma 3.33.1’ [35]. Only genes presenting a fold change value (FC) ≥ 1.5 and a P Value < 0.05 were selected, as differentially expressed.

In addition to the univariate comparisons between VTSW and Control’s conditions, an unsupervised multivariate analysis (sPCA) based on all genes was performed to compare the conditions in a more global approach [36]. The analysis was performed using the SPC function of the PMA ‘R’ package (v3.3.1).

Thematic analysis of over-represented groups of genes

Functional analysis of differentially expressed genes was carried out by Laboratoires StratiCELL S.A. (Isnes, Belgium) using the ‘StratiCELL Skin Knowledge Database’ [37], Straticell’s proprietary database in which the genes are annotated in a context of cutaneous biology, based on public databases knowledge. The enrichment or over-representation of functional groups was carried out with the hypergeometric test method.

PCR for gene expression modulation validation

We used pRT-PCR to quantified genes of interest selected by microarray analysis. The PCR was normalized with two housekeeping genes (B2M-b-2-microglobulin, and YWHAZ-tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) and was carried out using ‘TaqMan Gene Expression Assays’ (Applied Biosystems) with the 7900HT Fast Real-Time PCR (Applied Biosystems). The list of the genes quantified and the Applied Biosystems primers references are listed in Table II.

Immunofluorescence

NHEK were isolated from foreskin leftovers and cultured in EpLife™ medium at 37 °C for 3 days, then transferred into a powdered
Table II: Primers list for applied biosystems qRT-PCR

| Symbol | Gene Name                                                                                     | AB references |
|--------|-----------------------------------------------------------------------------------------------|----------------|
| B2M    | B2-microglobulin                                                                              | Hs00984230_m1 |
| YWHAZ  | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide        | Hs00442821_g1 |
| AQP3   | Aquaporin 3 (Gill blood group), Gill blood group                                               | Hs00185020_m1 |
| CAT    | Catalase                                                                                      | Hs00196308_m1 |
| CLDN1  | Claudin 1, senescence-associated epithelial membrane protein 1                                 | Hs00228163_m1 |
| COL7A1 | Collagen, type VII, alpha 1, collagen VII, alpha-1 polypeptide, LC collagen                    | Hs00164310_m1 |
| CTSV   | Cathepsin V                                                                                  | Hs00952036_m1 |
| DSG1   | Desmoglein 1                                                                                 | Hs00355084_m1 |
| DSG4   | Desmoglein 4                                                                                 | Hs01125472_m1 |
| EXO1   | Exonuclease 1, RAD2 nuclease family member, homolog of S. cerevisiae exonuclease 1             | Hs01161900_m1 |
| FANCD2 | Fanconi anaemia, complementation group D2                                                         | Hs00276992_m1 |
| GCLM   | Glutamate-cysteine ligase, modifier subunit, gamma-glutamylcysteine synthetase                  | Hs00157694_m1 |
| GJB5   | Gap junction protein, beta 5, 31.1 kDa, connexin 31.1                                         | Hs01921450_s1 |
| GPX2   | Glutathione peroxidase 2 (gastrointestinal)                                                   | Hs01591589_m1 |
| HBE2F  | Heparin-binding EGF-like growth factor, diphtheria toxin receptor                               | Hs00181183_m1 |
| HMOX1  | Haeme oxygenase (decycling) 1                                                                  | Hs01102050_m1 |
| JUP    | Junction plakoglobin                                                                          | Hs00158408_m1 |
| KLK5   | Kallikrein-related peptidase 5                                                                 | Hs01548153_m1 |
| LAM7A3 | Laminin, alpha 3                                                                             | Hs00165042_m1 |
| LAM7B3 | Laminin, beta 3                                                                              | Hs00165078_m1 |
| LAMC2  | Laminin, gamma 2                                                                             | Hs01043711_m1 |
| MKI67  | Marker of proliferation Ki-67                                                                  | Hs01032439_m1 |
| RAD1   | RAD1 homolog (S. pombe), exonuclease homolog RAD1, checkpoint control protein HRAD1, cell cycle checkpoint protein HRAD1, RAD1-like DNA damage checkpoint TDNA repair exonuclease REC1 | Hs00984990_g1 |
| RAD18  | RAD18 homolog (S. cerevisiae)                                                                  | Hs00892551_m1 |
| RAD23A | RAD23 homolog A (S. cerevisiae), RAD23, yeast homolog, A                                      | Hs00908419_m1 |
| RAD51  | RAD51 recombinase, BRCA1/BRCA2-containing complex, subunit 5                                  | Hs00947967_m1 |
| RAD54L | RAD54-like (S. cerevisiae)                                                                    | Hs00891777_m1 |
| SPN    | Statrin1,14-3-3 sigma                                                                        | Hs00685671_s1 |
| SIRT1  | Sirtuin 1                                                                                    | Hs01009006_m1 |
| TOPBP1 | Topoisomerase (DNA) II-binding protein 1                                                       | Hs00199775_m1 |

McDB153 medium (Calcium free) solubilized in either pure water (with and w/o CaCl₂ at 1.8 mM or 200 mg L⁻¹) or VTSW at 25% and 50% for 24 h. NHEK’s were fixed and incubated with primary antibodies directed against TGM-1 (Harbor Bio Products 5003), CK-10 (Abcam ab76318) and FLG (Abcam ab81468) followed by appropriate fluorescent secondary antibodies (Molecular Probes A11001 or A11008). In addition, a fluorescent molecule, DAPI (4,6-diamino -2-phenylindole) allowed the detection of the nuclei of keratinocytes. The various fluorescent images, obtained from a Leica camera (DFC420C, obj. ×40), were analysed by dedicated software (QWin/Leica/6 images per culture).

**Results**

**Transcriptomic study**

**Unsupervised sparse Principal Component Analysis (sPCA)**

A majority of genes bring more noise than information and make the estimates of multivariate analyses unstable. In order to reduce the size of the matrix to a limited number of unsupervised selected genes, with minimal loss of information (low-rank reconstruction), a sPCA with a Lasso constraint has been performed using the normalized expression data for all the genes measured on the microarray. This powerful and robust approach allowed to reduce the dataset to 2862 genes. A representation of the samples in the first factorial plane shows a clear separation between the samples associated with the thermal water and the control growth medium on the first factorial axis (Fig. 1).

This robust unsupervised approach demonstrates clearly that the samples from the untreated and TVSW treated conditions present clear differences in their gene expression patterns.

**Supervised analysis: differentially expressed genes**

When keratinocytes were grown in the presence of 50% VTSW for 18 h, the expression of 1745 probesets (including 1356 annotated probesets) was found modulated, with a threshold of significance set for a P-value P < 0.05 and a fold change |FC| ≥ 1.5. The thematic analysis of these selected genes exhibiting a |FC| ≥ 1.5 through Stratificl proprietary skin database revealed that 34 modulated genes were representative of functional groups particularly relevant of skin biology, namely structure of DEJ, epidermal renewal, cell-ECM and cell-cell interactions, and desquamation (Fig. 2, Table S1). 14 modulated genes were linked to the response to oxidative stress (Fig. 3, Table S2), and 12 modulated genes were involved in the response to stress-induced DNA damage and DNA repair (Fig. 4, Table S3). A few genes with 1.3 ≤ FC ≤ 1.5 were included in supplementary Tables I and II, when directly linked to the modulated functions.

For key genes selected for epidermis structure, oxidative stress response and stress-induced DNA damage, qRT-PCR analysis...
confirms the change of their expression in response to VTSW (Table S1, S2, S3).

Impact of VTSW on epidermis structure-related gene expression

The expression of a set of 34 genes was found modified (Fig. 2, Table S1).

Components of dermal-epidermal junction. The expression of the three subunits LAMA3, LAMB3 and LAMC2 of laminin 332 (LAMA3 showed a weaker FC, 1.3) was coordinately triggered, together with the two subunits of its cognate receptor, α6β4 integrin [38]. Nidogen-1, a key basement membrane organizer [39] and collagen VII, a key component of anchoring fibrils, were also found increased but with FC < 1.5 (1.3 and 1.4, respectively).

Cell-ECM interactions. Several ECM receptors were induced (ITGA 2, 4, 6 and ITGB4) as well as components of focal adhesion (CAV1, VCL).

Epidermal proliferation–differentiation balance. The expression of two key markers of cell proliferation, namely Ki67 and PCNA (Proliferating Cell Nuclear Antigen) was found increased, together with that of AQP3, CD44 (FC = 1.3), HBEGF and SFN. Induction of AQP3 was confirmed by qRT/PCR (FC = 4.3, P < 0.001). Of note, AQP3 is expressed in the basal and spinous epidermal cells, and is the most abundant skin aquaglyceroporin. Recent data suggest that glycerol transport by AQP3 is involved in the metabolism of lipids in skin as well as in the regulation of proliferation and differentiation of keratinocytes [40, 41]. CD44 is involved in the regulation of keratinocyte proliferation in response to extracellular stimuli [42]. HBEGF is a keratinocyte autocrine factor which plays a major role in the skin healing process by accelerating the migration of keratinocytes during re-epithelialization phase and contributing to a faster closing of the wound [43]. Stratifin, or 14-3-3 protein, is involved in the epidermal proliferation–differentiation switch [44].

Cell–cell interactions. Most of the desmosome components were induced, including desmogleins (DSG1, 4), Plakoglobin (JUP), plakophilins (PKP) 1.2 and to a lesser extent desmocollin 3 (DSC3) (FC = 1.3) [45]. Of note, DSC1 expression was decreased. Besides, components of tight junction (CLDN1) [46] and GAP junction (GJB3/Cx31 and GJB5/Cx31.1, and to a lesser extent GJB4/Cx30.3 with FC = 1.4 and GJA1/Cx43 with FC = 1.43) [47] were triggered.

Stratum corneum maturation. In the presence of 50% VTSW, genes encoding proteins playing a key role in the desquamation process were found overexpressed, namely kallikreins 5 and 7 (KLK5 and KLK7), cathepsin V and D (FC = 1.4). Moreover, Cystatin M/E (CST6), an inhibitor of the cathepsins, is downregulated. KLK5 and 7 both degrade corneodesmosomal proteins [48], Cathepsin D degrades desmosomes during epidermal desquamation [49], whereas Cathepsin V, together with Cathepsin D [50], controls the cleavage and activation of transglutaminase-1 (TGM1) and transglutaminase-3 (TGM-3) and thus the cross-linking and desquamation of the stratum corneum [51].

Response to oxidative stress

Besides the above 34 genes directly linked to epidermal proliferation–differentiation balance, a set of 14 genes stimulated in the presence of 50% VTSW: these genes are directly linked to the oxidative stress response (Fig. 3, Table S2), and more specifically to the Nrf2 pathway, the redox status, and iron storage.

Five genes were identified targets of nuclear factor erythroid 2-like 2 (Nrf2), a basic leucine zipper transcription factor that binds to the promoter sequence ‘antioxidant responsive element (ARE)’ leading to coordinated upregulation of ARE-driven detoxification and antioxidant genes [52]. Although 50% VTSW had no stimulatory effect on Nrf2 expression itself in keratinocytes, it stimulated the expression of NQO1, HMOX1, GCLM, G6PD and, to a lesser extent, TXRND1 (FC = 1.3). NQO1 encodes the NAD(P)/H Quinone Dehydrogenase 1 that promotes the reduction in quinones to hydroquinones, thus preventing the production of radical species. NQO1 is overexpressed after contact with pro-oxidant agents and heavy metals, after exposure to UV or ionizing radiation [53]. HMOX1 codes for the haeme oxygenase (HO-1), known to be rapidly induced post-exposure to free radicals (ROS), and for its protective role against oxidative stress [54]. HMOX1 induction by VTSW was confirmed by qRT/PCR (FC = 83.1, P = 0.001). TXRND1 codes for the thioredoxin reductase 1, an enzyme catalysing the transfer of electrons from NADPH to the thioredoxin, which itself has a reducing activity towards various targets, including peroxyredoxine proteins. Interestingly, we observed a down-regulation of TXNIP expression, which is an inhibitor of...
thioredoxin reductase 1 activity [55]. G6PD is a key enzyme involved in the maintenance of intracellular level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). Of note, in response to 50% VTSW, keratinocytes overexpressed 2 members of the peroxiredoxine family, namely PRDX1 and 3. This thioredoxin system is one of the major antioxidant defense systems which maintains glutathione status [56]. In this respect, GCLM encodes the catalytic subunit of glutamate-cysteine ligase, an enzyme essential for the synthesis of glutathione [56], a key factor in the maintenance of cell redox potential and in a number of reactions of detoxification and elimination of reactive oxygen species. The genes encoding GSR and GPX2, 2 enzymes essential to this system are also overexpressed in the presence of VTSW. GSR, glutathione reductase, restores the pool of reduced glutathione from its oxidized GSSG form [57], whereas GPX2, Glutathione peroxidase 2, is a selenoprotein which catalyses glutathione oxidation (GSH to GSSG) and thus ensures the transformation of organic hydroperoxides and H₂O₂. GPX2 induction by VTSW was confirmed by QRT/PCR (FC = 11.43, P = 0.002). To a lesser extent, Catalase (CAT) was also induced (FC = 1.32) in the presence of 50% VTSW (FC = 1.3). By decomposing H₂O₂ into H₂O and O₂, CAT is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS) [58]. Finally, a joined stimulation of the genes coding for both heavy and light ferritin chains (FTH1 and FTL1) was noted. Ferritin is often overexpressed as a result of exposure to a free radical stress. It stores iron resulting from haeme degradation, in a not harmful form [59].

Response to stress-induced DNA damage

The expression of a set of 12 genes (BRIP1, EXO1, FANCD2, RAD51, RAD54L, RAD23A, RAD1, RPA1, RAD18, TOP3B, TOPBP1 and SIRT1), all involved in the response to stress-induced DNA damage and DNA repair was increased in the presence of 50% VTSW (Fig. 4, Table S3). BRIP1 is a member of the RecQ DEAH helicase family and, by interacting with BRCA1, is important in the normal
double-strand break repair function. FANCD2, RAD51 and RAD54L are involved in homologous recombination of DNA during double-strand break repair. EXO1 is involved in DNA mismatch repair, whereas PCNA acts as a scaffold to recruit proteins involved in DNA replication and DNA repair. RAD1 encodes a component of a heterotrimeric cell cycle checkpoint complex, known as the 9-1-1 complex, which is activated to stop cell cycle progression in response to DNA damage or incomplete DNA replication. RPA1 binds to ssDNA during the initial phase of homologous recombination, an important process in DNA repair and DNA damage checkpoint activation. RAD18 is involved in post-replication repair of UV-damaged DNA, whereas RAD23A is involved in nucleotide excision repair (NER). TOP3B plays a role in DNA recombination, cellular ageing and maintenance of genome stability. TOPBP1 is a key scaffold protein that links crucial components involved in DNA replication initiation, DNA-dependent checkpoints, DNA repair and transcription. Finally, SIRT1 has been shown to deacetylate and thereby deactivate the p53 protein and inhibits NF-κB-regulated gene expression by deacetylating the RelA/p65 subunit of NF-κB at Lysine 310 [60].

We chose to check the modulation of expression of nine genes with qRT/PCR. Except for SIRT1 (FC = 1.3), the stimulation of eight other selected genes linked to stress-induced DNA damage, was confirmed by qRT/PCR (Table S3).

**Phase contrast microscopy and immunofluorescence study**

Transcriptomic results indicated that VTSW may modulate the proliferation–differentiation balance in keratinocytes, by stimulating the terminal differentiation pathway. To further characterize this effect, VTSW was compared to Ca ++, a well-known epidermal differentiation factor. As expected, phase contrast microscopy clearly demonstrated that at low calcium (control), NHEKs proliferate and did not differentiate, as shown by numerous replicating cells and the homogeneous non-adherent and refringent aspect (Fig. 5). On the opposite, a strong pro-differentiating effect was observed in the presence of 1.8 mM CaCl$_2$, as shown by strong cell packing, small size, adhering and flattened aspect, with local piling. Of note, an intermediate pro-differentiating effect was observed in the presence of 25% and 50% VTSW, some proliferating cells being still detectable (Fig. 5, arrowheads). In addition, and as expected [25, 26], immunofluorescence detection demonstrated that TGM-1 and CK-10 expression was strongly stimulated when NHEKs were grown in the presence of 1.8 mM CaCl$_2$, (Fig. 6). FLG expression was, however, only slightly increased under a non-significant manner. On the other hand, when NHEKs were grown in the presence of VTSW, a dose-dependent and significant increase in all three differentiation markers were observed, suggesting that the VTSW ionic...
mix may have a stronger effect on keratinocyte differentiation than CaCl2 alone (Fig. 5, Table S4). One thus cannot exclude that other ions, such as potassium (Table I), cooperate with calcium at controlling terminal differentiation [28].

**Discussion**

Various cations are known to being involved in a number of biochemical and biological processes. Bone mineralization and haemoglobin activities are obviously the most striking examples, but the role of Ca²⁺ gradient in epidermal differentiation is also well established [25,26]. It was thus quite legitimate to further study the potential effect of a spring water with a high minerality on human keratinocyte homeostasis, proliferation and differentiation. VTSW was chosen, because of its specific high content of mineral cations and specifically calcium (see Table I). A full genome transcriptomic approach was chosen in order to cover all possible pathways possibly sensitive to VTSW, without any preconceived bias. This study was performed on foreskin NHEKs, which are considered as bona fide human keratinocytes and are commonly used in studies dealing with terminal differentiation and more specifically the role of calcium [61,62]. Unexpectedly, a vast array of biological processes was modulated by VTSW, and more interestingly, under a coordinate fashion.

**VTSW and age-related DEJ alterations**

Alteration of the DEJ is a landmark of skin ageing, with delamination of the basement membrane, and the decrease in expression of collagen VII [5], laminin 5 and α6β4 integrin [6], among others. In the presence of VTSW, the expression of collagen VII, laminin 5 and α6β4 integrin was coordinately stimulated, together with nidogen 1, a key basement membrane organizer [39]. By re-enforcing the basement structure and the keratinocyte adhesive capacity to it, VTSW thus appears as an ingredient endowed with a capacity of restoring DEJ integrity.

**VTSW and epidermal renewal**

Alteration of the proliferation–differentiation balance is another landmark of skin ageing [63]. Interestingly, in the presence of VTSW, the expression of two reference markers of cell proliferation, namely Ki67 and PCNA, was increased as well as that of heparin-binding EGF-like growth factor (HBEGF), a keratinocyte autocrine growth factor involved in wound re-epithelialization [43]. Of note, modulators of the proliferation–differentiation switch were also induced, such as CD44, stratifin and AQP3 [42, 44, 64]. This suggests that in the presence of VTSW, keratinocyte proliferation is stimulated, but under the control of key actors of

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**Figure 4** Spider graph describing the effect of VTSW on the expression of stress-induced DNA damage and DNA repair. The gene expression ratio between VTSW treatment and the untreated control are represented with the continuous line. The dashed line is the baseline representing no expression modulation (ratio = 1). Genes squared have been checked with RT-PCR: (i) bold and underline for genes with ratio ≥1.5 or ≤0.67. (ii) *: pV < 0.05.
the keratinocyte proliferation to differentiation transition, this latter process being essential for the maintenance of epidermal homeostasis. Of note, the stimulated expression of CK10, TGM-1 and FLG, as detected by immunofluorescence, confirms that the activation of proliferation and differentiation is indeed balanced in the presence of VTSW.

Figure 5 Effect of VTSW on cell morphology (phase contrast). HuKHN were incubated in control growth medium, in the presence of 25% VTSW, 50% VTSW, or 1.8 mM CaCl₂. Black arrowheads indicate replicating cells.

Figure 6 Effect of VTSW on TGM-1, CK-10 and FLG expression. HuKHN were incubated in control growth medium, in the presence of 25% VTSW, 50% VTSW or 1.8 mM CaCl₂.
VTSW and epidermal cohesion

Vichy Thermal Spring Water may play a role in epidermal homeostasis restoration (see above). However, part of this homeostasis maintenance is also the quality of cell-cell adhesion and communication. In the presence of VTSW, the expression of all the molecular entities of desmosomes was stimulated, desmosomes being the key structures in keratinocyte adhesion and epidermal integrity. The fact that DSC3 expression was increased and that of DSC1 decreased suggests that VTSW rather stimulated the formation of spinous layer desmosomes [44]. Besides desmosome components, a number of gap junction connexins were induced (namely GJA1 and GJB3, -4, and -5), suggesting that in the presence of VTSW, cell-cell communication and small metabolites’ transfer was stimulated, supporting cell synchronization, differentiation, cell growth, and metabolic coordination [47]. It is noteworthy that increased expression of DSG1, DSG4, GJB3, GJB4, and GJB5 is compatible with a stimulated keratinocyte differentiation, as all five isoforms are specific for stratum granulosum [45, 47]. Besides, the increased expression of CLDN1 tight junction protein suggests that in the presence of VTSW, not only keratinocyte mechanical stability is sustained but also the skin barrier function [47, 65]. In the latter respect, the stimulated expression of AQP3 could also contribute, as both water and glycerol transport by AQP3 appears to play an important role in hydration of mammalian skin epidermis [40, 66], AQP3 appearing as a prime target to combat the skin dryness associated with age and the repeated exposures to UV and to restore an effective skin barrier [66].

Altogether, VTSW appears as an ingredient endowed with a capacity to improve epidermal cohesion, mechanical properties and internal communication, and ultimately barrier function.

VTSW and Stratum corneum formation

Besides specialized adhesion structures (see above), epidermal barrier function mostly relies on a proper stratum corneum maturation and desquamation. Again, it is striking to note that in the presence of VTSW, the expression of two key enzymes (Cathepsin V and L) involved the activation of Tgase 1 and 3 [50], was stimulated, thus favouring cross-linked envelope formation. Under a coordinated fashion, Cathepsin D, KLK5 and KLK7 expression were also increased. Of note, these enzymes are involved in stratum corneum desquamation process. However, the increased expression of Cystatin M/E, an inhibitor of cathepsins [51], suggests that, if cross-linked envelope formation and desquamation may be triggered in the presence of VTSW, it would be under a controlled and balanced way.

VTSW and response to oxidative stress

In the presence of VTSW, a block of 14 coordinately stimulated genes are noted, five of them corresponding to antioxidant enzymes regulated by the Keap1-Nrf2 pathway [56], covering different antioxidant mechanisms. Among these, prevention of radical species production (NQO1), activation of the thioredoxin system (TXNRD1) (one of the major antioxidant defense systems) linked to peroxiredoxins’ activation (PRDX1,2,3 and 6) [67], reduction (GPX2) and dismutation of H2O2 (CAT) are potentially activated in keratinocyte response to VTSW. Of note, the activation of catalase (CAT) was previously measured in the stratum corneum, after in vivo application of VTSW to human skin [30]. However, considering the weak induction of in vitro CAT gene expression (not confirmed by qRT/PCR, Table S2), this in vivo CAT activation by VTSW was more likely linked to the VTSW high hydrogenocarbonate content and buffering effect around pH 7.0. Indeed, the activity of native catalase is severely suppressed by acidic condition close to that of skin surface (pH 4.5–5.5) and restored at neutral pH [68]. In parallel, the expression of two key enzymes of glutathione cycle, namely GSR and GPX2, is also stimulated, together with that of GLCM, a key enzyme in glutathione synthesis pathway. Altogether, an impressive array of antioxidant mechanisms is potentially mobilized in the presence of VTSW, under a coordinated way, positioning VTSW as an ingredient that may help to tackle oxidative stress, a major factor of skin ageing exposome [18].

VTSW and Response to stress-induced DNA damage and DNA repair

The expression of a set of 12 genes involved in DNA repair and response to stress-induced DNA damage was increased in the presence of 50% VTSW. The expression modulation of nine of these genes was further confirmed by qRT/PCR (Table S3). These genes encode for enzymes involved in double-strand break repair function (FANCD2, RAD51 and RAD54L), mismatch repair (EXO1), nucleotide excision repair (RAD23A), blockade of incomplete DNA replication (RAD1), post-replication repair (RAD18) and DNA checkpoint progression (RPA1, TOPBP1). VTSW may thus support the keratinocyte response to DNA damage by activating DNA repair mechanisms, key processes involved in natural skin protection against harmful effects of the solar UV, another major factor of skin ageing exposome [18]. In parallel, increased expression of SIRT1, though weakly increased (FC = 1.3 by qRT/PCR, Table S3), may also be of importance by inhibiting NF-κB-regulated gene expression [60], human ageing being characterized by a chronic, low-grade inflammation level and a sustained activation of NF-κB signalling pathway [13].

VTSW and skin ageing exposome

The expression of 79 genes is modulated – and for the vast majority, stimulated – in keratinocytes grown in the presence of 50% VTSW. Two main features characterize this transcriptomic effect: (i) these genes are clustered in functional groups, which cover main processes involved in skin homoeostasis, skin ageing and response to skin ageing exposome and (ii) for a given function, most if not all molecular entities are involved (all connexins, all desmosomal components, entire thioredoxin/peroxiredoxine pathway, etc…). These coordinated and integrated responses to VTSW might be linked to the key – and sometimes neglected – role(s) of cations in regulating and co-activating a whole array of enzymes and epidermal functions which are altered during skin ageing and by skin exposome.

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Conflict of Interest

All authors are employees of L’Oréal Company.
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