Research Article

Dermatology Research

Hard Water and Cleansers: An *In-Vitro* Study of the Effects on Immature Reconstructed Human Epidermis

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ABSTRACT

Background: *Barrier function impairment resulting from increased exposure to daily use of surfactants in the presence of hard water (HW) has been identified as a relevant factor to the development of atopic dermatitis (AD), especially in infants.*

Objectives: *To investigate the effect of HW as such or in the presence of surfactants on skin barrier development as it occurs during infancy, using a 3D reconstructed immature (not fully differentiated) human epidermis model.*

Materials and Methods: *Reconstructed Human Epidermis (RHE) starting at the 13th day of differentiation was exposed to HW alone or in the presence of surfactants (Sodium Dodecyl Sulphate and a commercial cleansing oil) for 2 days (two topical exposure per day) followed by two days of recovery. The barrier structure and functional modifications have been assessed by evaluating epidermal morphology (H&E), tight junctions barrier functionality (TEER measure), proteins' expression of claudin-1, filaggrin and involucrin by immunohistochemistry, inflammation by transcriptional activity of TNF-α and RHE ultrastructure (TEM analysis).*

Results: *HW itself has modified the differentiation process without influencing the barrier function structure (Claudin 1 was not significantly affected) but in presence of SDS, HW has induced inflammation, morphological modification in particular cell swelling and SC lamellar structure damages leading to a severe modification to the barrier structure in the final epidermal differentiation. The effects of the hard water are reduced in the presence of a mild cleansing oil including emollients.*

Conclusion: *The results obtained using the in-vitro RHE model are in agreement with the clinical observations reported in the literature regarding the detrimental effect of the hard water on the skin of infants. Moreover, the in-vitro model can be a useful tool to evaluate the effects of both surfactant and cleansing formulation on impaired skin.*

Keywords

Hard water, Cleansers, Atopic dermatitis.

Introduction

Atopic dermatitis (AD) is an inflammatory skin disease, which manifests itself primarily during infancy, affecting significantly both children and parents' life [1]. About 20% of children worldwide suffer from AD and this number is growing both in industrialized and developing countries [2].

AD aetiology is complex and multifactorial: skin and immune dysfunctions, environmental factors, and genetic predisposition, all play a role in its development and are closely intertwined. The differences within countries point out the importance of environmental factors such as pollution, climate, diet and hygiene's routine in AD development [3]. Infant's skin is more affected by all these elements because it differs from adult's one in many aspects as observed at ultrastructural level and by immunocytochemical corneocyte labelling: the *stratum corneum* (SC) of infants is 30%

thinner, the process of corneocyte desquamation is less regulated, and water-handling properties are not fully efficient [4]. For all these reasons infant's skin is more fragile and susceptible to the environment and every-day hygiene procedures.

Among the external factors, daily cleansing routine with body wash plays a crucial role directly linked to surfactants chemical structure, their relative concentration, and their skin irritation potential which leads to SC proteins denaturation and imbalance of moisturization mechanisms, determining a further impairment of the fragile barrier of subjects affected by AD. Recent studies have highlighted that also the domestic water used in daily care has an important role in the development and/or progression of atopic dermatitis [5,6]. In the last years, the scientific world has started to pay attention to the hardness of water as one of the triggering factors of atopic dermatitis indeed. Domestic water is characterized by a certain level of hardness which is determined by the concentration of calcium and magnesium carbonates: higher is this concentration, higher is water hardness. This parameter depends on the geology of the territory, so it is characteristic of each living area. Perkin MR et al. reported that three-month-old infants living in high water hardness areas were more likely to develop AD compared with those living in low water hardness areas. The authors suggest that carbonates contained in water could have a harmful effect on the skin barrier integrity and this results in an increased dryness and development of eczematous skin inflammation. Another highlighted factor is that water with a high concentration of carbonates has higher pH, which leads to an increase of pH on and in the SC. This leads to higher protease activity, which accelerates the disruption of the corneodesmosomes and reduces lipid lamellae synthesis: the final effect is the breakdown of the skin barrier [5].

Danby SG et al. proposed another mechanism by which high calcium levels contained in hard water can increase the risk of developing AD. They found that hard water reduces the solubility of surfactants, in particular ionic surfactants, such as *sodium lauryl sulfate*, which deposit on the skin after rinsing. Surfactants residue alters protein secondary structure, solubilizes SC lipids and increases skin surface pH. All these effects result in increased TEWL and cause irritation and skin barrier impairment, particularly in AD patients carrying FLG mutation compared with individuals with AD without FLG mutations and individuals with healthy skin [6]. Thus, barrier impairment resulting from increased exposure to surfactants in the presence of HW can be considered a contributory factor to the development of AD, especially in infants.

Mild dermo-pharmaceuticals products are part of the management of AD both for young and adult patients since they should guarantee the absence of negative effects of surfactants on the barrier structure and function after repeated exposures. The effective mildness of a cleanser depends on its composition and so under the umbrella of mild labelled cleansers there are products with no impact on the skin barrier and others which can alter it. Since both hard water and cleansers are recognized as two pivotal factors in worsening AD condition there is a need for predictive pre-clinical experimental models to identify the effect of the hard water, alone and added of cleansers, on immature skin.

In this work is described the development of an in-vitro 3D model of immature reconstructed human epidermis (RHE) that aims to gain a better understanding of the effect of HW and cleansers on the not-fully-differentiated skin barrier. We used the RHE tissues at the 13th day of differentiation in order to better mimic the fragile skin of the newborns which has a higher permeability as it occurs in AD. The model has been used to quantify the deleterious effects of exposure to HW (with high *calcium carbonate* content) alone or in presence of a reference surfactant (*sodium dodecyl sulfate*) and of a mild cleanser containing a polymeric surfactant and a mixture of ceramides, cholesterol and fatty acids. The barrier structure and the functional parameters were compared to water with a standard *calcium carbonate* content. The study aimed also at exploring if milder toiletries with emollients could guarantee a better recovery of barrier impairment induced by the cleaning procedures. The epidermal lipids (ceramides, cholesterol, fatty acids) are known to repair and improve the barrier function and could mitigate skin damages and allow a physiological and efficient formation of the barrier structure [7-9].

Methods

Test chemicals and Solution preparation

The hard water (40 f) used in the study was ultrapure water (obtained with Nex Power 1000 System - Fulltech) supplemented with *calcium carbonate* (400 mg/L). Ultrapure water, with a standard *calcium carbonate* content, has been used as the negative control. *Sodium dodecyl sulfate* (CAS 151-21-3) obtained from Sigma-Aldrich (St. Louis, Missouri, United States) has been used as the reference of ionic surfactant, positive control.

The mild cleanser has been supplied by Unifarco Biomedical (Santa Giustina BL, Italy). It is a detergent emulsion that can be used with or without water, containing a synthetic emollient (Octyldodecanol) and a non-ionic polymeric surfactant (Poloxamer 188) as detergent agent. It is also enriched with epidermal lipids (Ceramide 3, Cholesterol and Stearic Acid) as repairing agents.

The test dose for the product was 10 µL of a solution at 0.05% (0.5 mg/mL) twice a day. Thus, solutions at 0.05% of *sodium dodecyl sulfate* (SDS) and mild cleanser have been prepared by dissolving them in HW and then applying the solution to the tissues (volume 10µL). In Table 1 is shown the list of samples and codes.

Sample	Description	Code
Negative control	Ultrapure water with standard *calcium carbonate* content	NC
Hard Water	Ultrapure water supplemented with *calcium carbonate* (400 mg/L)	HW
Sodium Dodecyl Sulfate	0.05% *sodium dodecyl sulfate* in hard water	HW + 0.05% SDS
Mild cleanser	0.05% mild cleanser in hard water	HW + 0.05% mild cleanser

Table 1: List of samples and respective codes.

Biological System

SkinEthic RHE model of 0.5 cm^2 from EPISKIN (Lyon, France) has been received at day 12 when the epidermal differentiation process is not fully achieved (it is considered differentiated at day 17) and the barrier can be considered immature.

Exposure Conditions

In order to mimic a realistic daily personal hygiene procedure and considering the features and barrier properties of reconstructed tissues, a twice-daily application of 10 µL of the product has been chosen. From day 13 to day 14, 10µL of HW, 0.05% of SDS and 0.05% of the mild cleanser both in HW have been directly and uniformly applied topically on the epidermis surface twice a day. This corresponds to 4 applications in 2 days. Then, the tissues have been maintained in culture at 37°C and 5% CO_2 for recovery until day 17, which is considered the reference time for a fully differentiated RHE model. Before the second product application at days 13 and 14 and before the first daily application at day 14, residuals volumes are removed by suction but not washed. At the end of treatment (day 14 and day 17), tissues are rinsed with 2 mL saline before measurements and analysis.

Multiparametric analysis

The in-vitro testing on RHE tissues allows evaluating several parameters with different analytical approaches leading to a multiple endpoints analysis approach. In this study, it includes: histo-morphological analysis (Haematoxylin and Eosin staining - H & E); claudin 1, involucrin and filaggrin immunostaining; transcriptional activity of TNF-α gene by qRT-PCR; measurement of a functional parameter of tight junctions efficiency (trans-epithelial electrical resistance - TEER); ultrastructural tissue analysis by Transmission Electron Microscopy (TEM).

As reported in Table 2, these endpoints have been evaluated at day 14 (4 exposures in 2 days) and day 17 (4 exposures followed by three days of recovery) for all the samples listed in Table 1.

	Day 14: IMMATURE EPIDERMIS (4 exposures in 2 days)		
TEER (t= 0)	TEER	PCR	H&E/IF
	Day 17: FULLY DIFFERENTIATED EPIDERMIS (4 exposures + 3 days recovery)		
TEER (t= 0)	TEER	H&E/IF	TEM

Table 2: List of endpoints tested after 4 exposures (day 14) and 4 exposures followed by three-day recovery (day 17).
Experiments were conducted in triplicate RHE tissues.

Histo-morphological analysis

At the end of the treatment and after product removal (day 14 and day 17) tissues have been fixed in buffered 10% formalin (Sigma-Aldrich). The samples have been included in paraffin (Histoline) blocks and sections of 5 µm have been cut. The slides were stained with H&E (Histoline) and analyzed under light microscopy (40x, LEICA DM 2500). The overall morphology and its modification compared to the negative control have been inspected on 3 non-consecutive sections of the same tissue. On one selected section 3 microscopical acquisitions were performed.

Immunofluorescence: Claudin-1, Involucrin, Filaggrin

RHEs have been fixed in 10% formalin and then tissues were embedded in paraffin.

RHEs have been rinsed and the aspecific reactions have been blocked using 1% BSA for filaggrin and claudin-1 and Protein Block (DAKO) for involucrin. Every insert has been incubated overnight at 4°C with the primary antibody, rabbit anti-human filaggrin (Sigma, HPA030188), anti-human involucrin (SIGMA HPA055211), anti-human claudin (Life-Technology, 51-9000). After rinsing, the tissues have been incubated at room temperature for 1 h with the secondary antibody (Alexa Fluor 555 donkey anti-rabbit, A31572, Life Technologies). After rinsing, the nuclei have been stained with DAPI and analyzed at the fluorescence microscope Leica DM 2500.

Real-time PCR

For all the steps, ready-to-use reagents have been used. The total RNA has been extracted from RHE tissues using the RNAqueous kit according to the manufacturer's protocol (Thermo Fisher Scientific, Italy). The cDNA has been then synthesized in a 20-µL reaction using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Italy) and subjected to reverse transcription in a thermal cycler (Thermo Fisher Scientific ABI 7500 Real-time PCR System) under the following conditions: 25°C for 10 min, 37°C for 60 min, 85°C for 5 sec. The same instrument with fluorescent-based PCR chemistry, the TaqMan assay, has been used to study gene expression of significant biomarkers. GAPDH has been used as an endogenous control gene to normalize input amounts. Each biological replicate has been assessed in triplicate. At the 2X TaqMan Fast Universal PCR Master Mix has been added Taqman gene expression assay and cDNA (25 ng) for a total volume of 20 µL. The Thermal condition steps in the ABI PRISM 7500 Fast have been: 95°C 20 sec; 40 cycles (95°C 3 sec +60°C 30 sec).

Fluorescence data of the RT-PCR generated by the thermocycler ABI 7500 Fast, have been collected by the internal software SDS 2.0. Because each cycle in the PCR reaction corresponded to a 2-fold increase in PCR product, a difference of one in threshold cycle number represented a 2-fold difference in the expression of a particular gene compared to the calibrator sample and could be considered significant. 95% of confidence level is used by the software to calculate the errors. Value has been accepted as significant when the gene is "one-fold" up (RQ>2) or down-regulated (RQ<0.5) compared to the calibrator sample (RQ=1).

Trans Epithelial Electrical Resistance (TEER)

To perform TEER measurements 0.5 mL of a saline solution have been directly applied on the tissue placed in a 6-well plate containing 5 mL of saline solution per well. The instrument Millicell-ERS has been placed with the electrodes in the two chambers. Three measurements for each tissue have been performed; the blank value (insert without tissue) has been subtracted to the sample value (mean of three measurements). This result has been then corrected considering the tissue surface (0.5 cm^2). TEER of each tissue has been measured at day 13 of differentiation before application and

at days 14 and 17 after product removal using the Millicell-ERS instrument (range 0-20 kΩ).

TEM analysis

After fixation in glutaraldehyde 2.5% and OsO4 1%, tissue samples were dehydrated in alcohol followed by propylene oxide and then embedded in epoxy resin (Durcupan). Ultra-thin section cutting is performed with Leica RM 2255 ultramicrotome. Visualization of the sections in TEM Microscopy is performed with Electronic Transmission Microscope (TEM) ZEISS LEO 912 AB.

Results

Histo-morphological analysis

As the first step, the RHE's morphology has been investigated.

In figure 1 are reported the H&E images of the RHE tissues treated differently and coded as reported in Table 1. The tissues have been collected at days 14 and 17 (immediately after the 4th exposure and after 3 days of recovery). In the NC the organization in four layers that form the epidermis is observed both at days 14 and 17 with increased thickness of the *stratum corneum* at day 17, as expected. The RHE exposed to HW appear damaged at both timepoints. In the late readout, the tissue morphology is severely modified showing the feature of a dry RHE: reduced viable epidermis thickness and damaged *stratum corneum* lamellar structure. RHE exposed to HW + 0.05% SDS at day 14 shows moderate modifications of morphology in the basal layer whereas significant modifications in the granular layer are visible with anucleate cells, pyknotic nuclei, and absence of keratohyalin granules.

At day 17, in the positive control (HW + 0.05% SDS) the early damages observed after exposure have determined significant modifications in all the viable layers: RHE appears necrotic and the *stratum corneum* has lost its regular staining and the organized lamellar structure. The RHE exposed to the mild cleanser is similar to the RHE exposed to ultrapure water at both timepoints, in fact, both on the viable epidermis and SC organization keratohyalin granules are visible. The modification of the basal layer near to polycarbonate filter observed at day 17 seems rather linked to a technical incident than to tissue damage. This preliminary observation on the overall morphology results supports the study rationale indicating HW as responsible for several modifications to

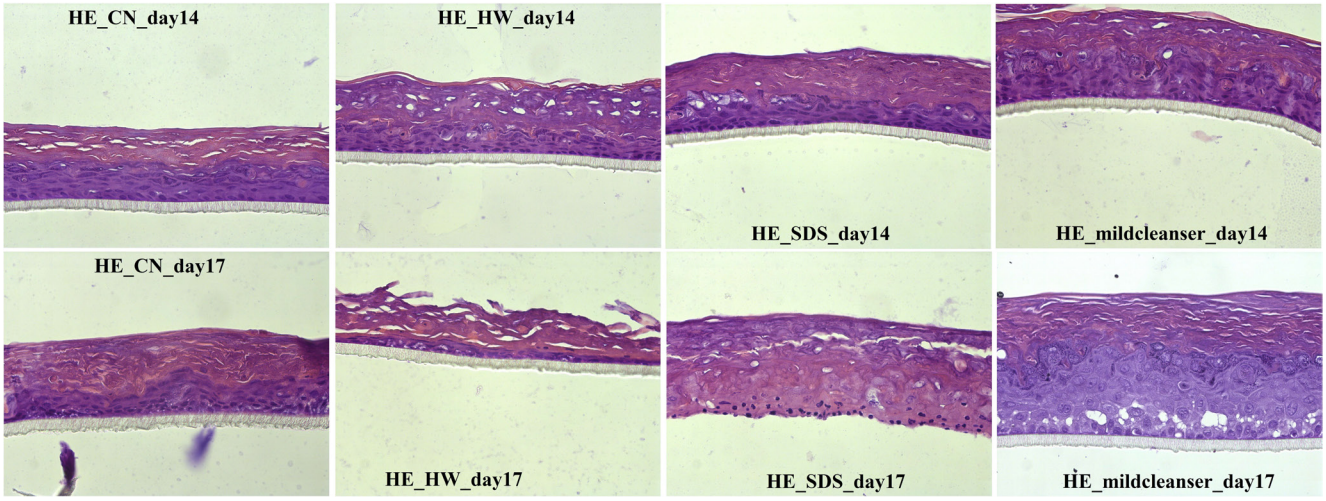

Figure 1: Modification of epidermal morphology in RHE at day 14 (corresponding to n= 4 exposures) and day 17 corresponding to 4 exposures + 3 days recovery.

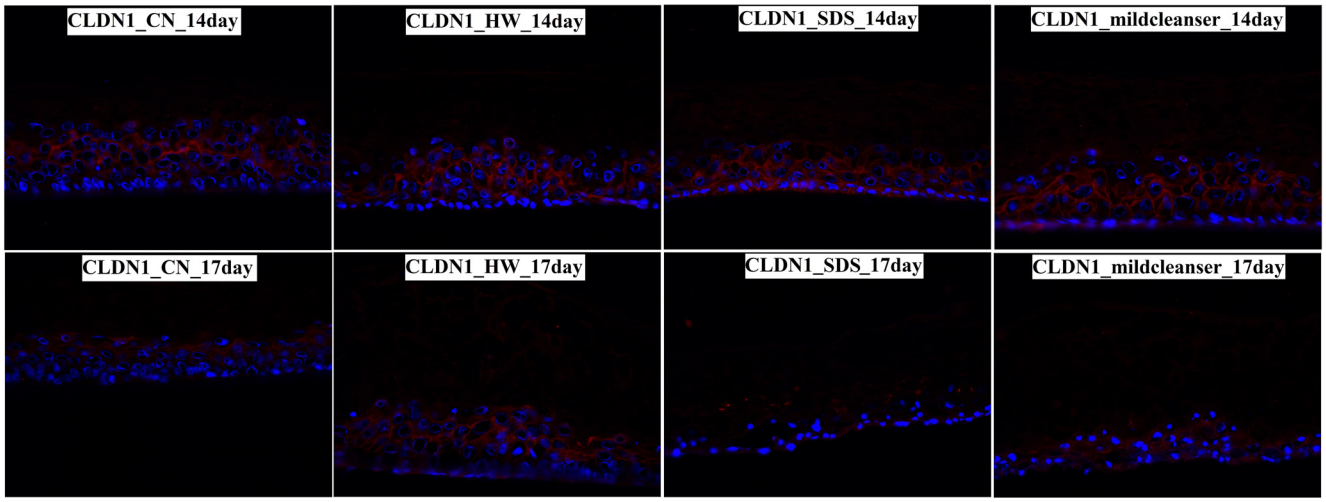

Figure 2: Claudin 1 immunostaining (red signal). Nuclei counterstaining with Dapi in blue at day 14 and day 17.

the epidermal barrier structure and that its activity is worsened in the presence of surfactants. The mild cleanser seems to counteract the HW negative effects allowing the expected development (epidermal differentiation) of the in-vitro RHE model.

Immunofluorescence assay

Claudin 1 (CLDN1) is one of the most important components of tight junctions (TJs): it limits the paracellular diffusion of small molecules through TJs in the epidermis, prevents excessive water loss maintaining water homeostasis and determines the normal barrier function of the skin [10].

In figure 2 are reported the images of CLDN1 immunostaining at day 14 and day 17. At day 14, in the NC the claudin 1 is present in the cellular membrane of supra-basal layers (from spinous to granulosum). The shape of the cells and their disposition in defined layers result regular, indicating a tight junction among cells and a correct organization of the tissue. At day 17, the overall CLDN1 staining results less intense in comparison to day 14, however, its distribution results regular in the upper stratum granulosum. The hard water has not modified the intensity of the CLDN1 staining at day 14, but the shape of the cells and their disposition in the epidermis result less regular indicating a possible loss of junction firmness. The same morphological modification is also evident at day 17 suggesting a significant detrimental effect of prolonged treatment with HW and confirming the H&E results. At day 14, the treatment with HW + 0.05% SDS has reduced the thickness of the tissue and flattened the cells: in the upper layers, the staining of the cell membrane seems to be faint in comparison with the NC and HW treatments indicating a possible early effect of barrier damage. At day 17, in SDS treated samples, no specific staining is visible indicating severe tissue damage. In the RHE exposed to the mild surfactant not significant differences from the negative control were observed. These results allowed to better describe the role of HW on epidermal differentiation and maturation as responsible for damages to TJs structure.

Involucrin and filaggrin proteins expression and localization were quantified at day 17, which is considered the time at which the full differentiation process of the in vitro RHE model is completed.

Involucrin as final differentiation biomarker leading to an efficient barrier function [11] has been investigated to demonstrate a possible impairment of epidermal barrier during its development (Figure 3). The signal was still visible in the RHE exposed to water with standard *calcium carbonate* content but its expression and localization in the upper epidermal layers was almost disappeared in the HW and HW + 0.05% SDS treated samples compared to the negative control. On the contrary, the signal was significantly higher in the sample treated with the mild cleanser.

Filaggrin is a key player in *stratum corneum* structure and formation enabling the primary function of the skin barrier to restrict water loss and to prevent the entry of irritants, allergens, and skin pathogens [12]. Loss-of-function mutations (of which more than 40 have been described) in FLG, which encodes filaggrin, have been implicated in up to 50% of patients with moderate to severe AD in some demographic populations [13,14].

Mutations in FLG are associated with a two- to three-fold increased risk of having AD [15]. The results (Figure 4) show a very clear filaggrin signal in the upper *stratum corneum* layers in the NC whereas in the HW or HW + 0.05% SDS treated RHEs the signal was no more visible: the negative filaggrin staining indicates the presence of damages to granular layer and on keratohyalin granules and confirms the H&E morphology results and the severe barrier defects. Filaggrin signal in RHE exposed to HW + 0.05% mild cleanser is consistent with a regular barrier structure and *stratum corneum* lamellar structure development.

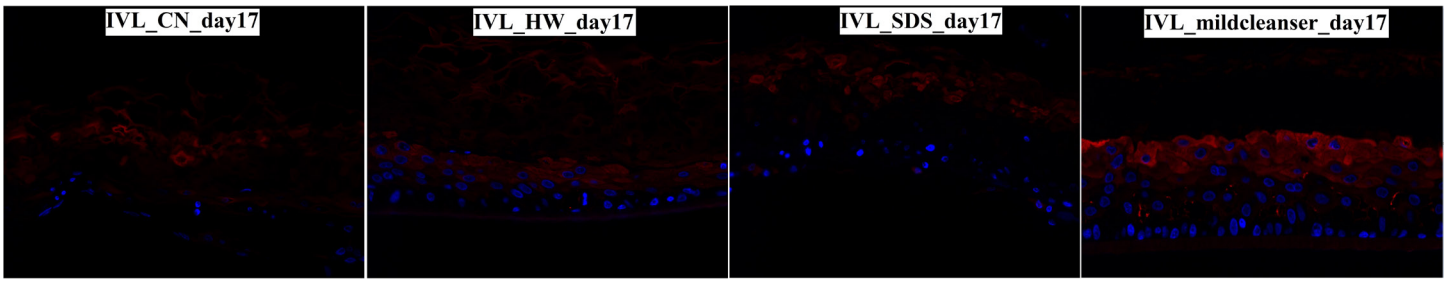

Figure 3: Involucrin immunostaining (red signal). Nuclei counterstaining with Dapi in blue at day 17.

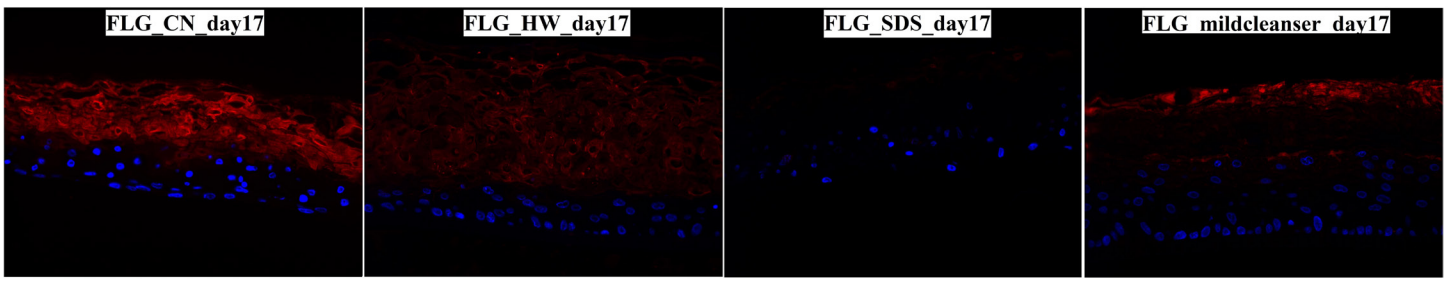

Figure 4: Filaggrin immunostaining (red signal). Nuclei counterstaining with Dapi in blue at day 17.

Gene expression of TNF-α

In order to assess the induction of inflammation, the expression of the TNF-α gene as a cytokine involved in the inflammation cascade, has been quantified by qRT-PCR in the RHE samples at day 14 as reported in Figure 5. The expression levels of TNF-α at day 14 indicate a strong pro-inflammatory effect of HW with 0.05% of SDS compared to HW with 0.05% of the mild cleanser, which does not induce a significant expression similar to the one induced by the HW alone.

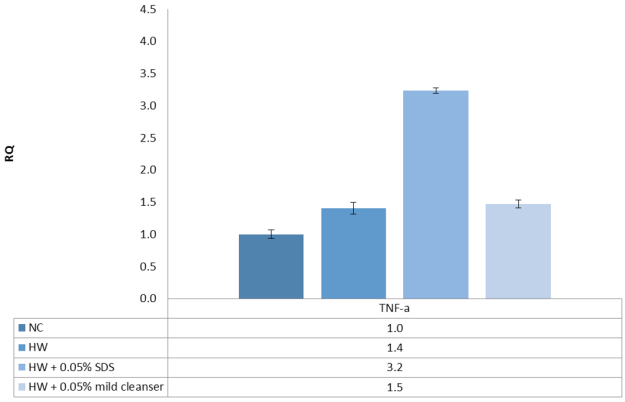

Figure 5: TNF-α gene expression level at day 14 by qRT-PCR. NC=RQ= 1.

Epidermal barrier functional measure: TEER measurement

The modification of the barrier properties has been assessed by comparing changes of the TEER values measured on RHE before (day 13, t= 0) and after treatment (days 14 and 17).

The mean TEER value recorded before treatment at day 13 for the RHE tissues employed for controls and product testing (N= 24) was 5820.42 ± 1479.89 Ohm*cm^2. In figure 6 are reported the normalized TEER values compared to the control values before treatment (day 13) considered as 100%.

TEER values were not significantly modified by the hard water alone despite the morphological modifications observed. TEER was found significantly decreased in the presence of HW + 0.05% SDS (2way ANOVA with multiple comparisons, $p \leq 0.0001$). The TEER reduction quantified for the surfactant solution and not for the hard water is due to the amphiphilic molecules which have a deeper impact on the epithelial permeability reducing RHE barrier properties.

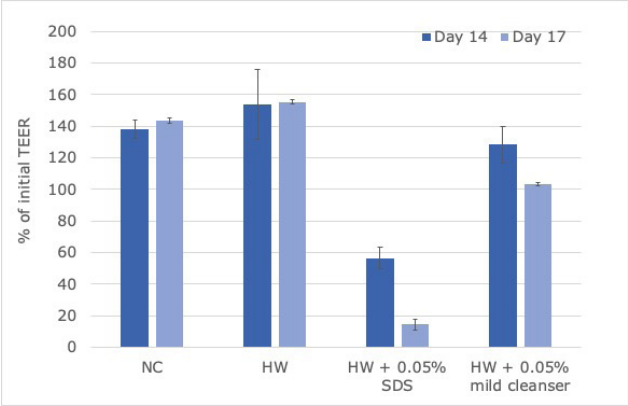

Figure 6: TEER values % with respect to TEER values of untreated tissues at day 13 considered as 100%.

The mild cleanser + HW has not significantly reduced the TEER values at day 14 but it has reduced the TEER in the post-incubation period compared to the negative control but with values significantly higher compared to SDS treated RHE.

TEM analysis

In figures 7-8 are reported TEM images of RHEs exposed to HW and the mild cleanser for 2 days (4 exposures) and then let them recover up (day 17). The TEM analysis shows that the mild cleanser has determined a production of keratohyalin granules which result more electrodense and large throughout the cellular cytoplasm (Figure 7).

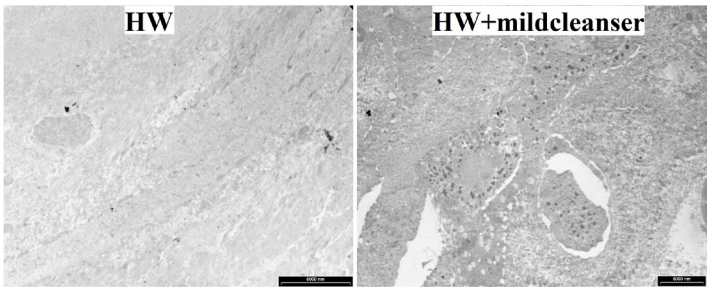

Figure 7: TEM images of RHEs exposed to HW and HW + 0.05% of the mild cleanser at day 17 showing the keratohyalin granules.

It is important to underline the absence of keratohyalin granules in the HW exposed samples leading to an imbalance in water retention capacity and moisturization. The keratinocytes globally show a high ribosome number and a more homogeneous distribution of tonofibrils (Figure 8).

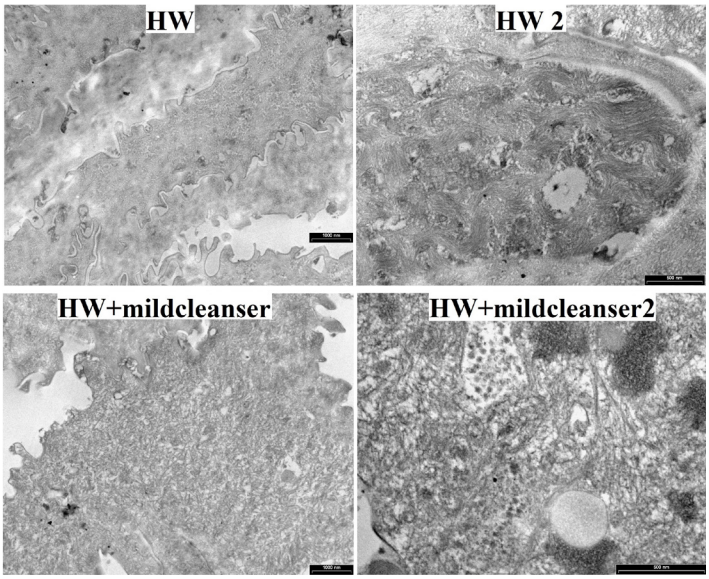

Figure 8: TEM images of the RHEs exposed to HW and HW + 0.05% of the mild cleanser at day 17 showing the ribosomes, tonofibrils, desmosomes, and the odland bodies.

Conclusion

The development of an in-vitro model of immature epidermis challenged by repeated exposures to hard water (with high *calcium carbonate* content) was presented. This model uses Reconstructed Human Epidermis (RHE) tissues at day 13 of differentiation to mimic fragile skin with higher permeability typical of the infants and the barrier development in infancy.

A Multiple Endpoint Analysis approach was adopted which includes the following endpoints:

- Epidermal morphology and viability by histo-morphological analysis (Haematoxylin and Eosin staining - H&E)
- Claudin, involucrin and filaggrin immunostaining
- Inflammation by TNF-α gene expression (qRT-PCR)
- Barrier integrity by trans-epithelial electrical resistance (TEER) measurement
- Ultrastructural tissue analysis by Transmission Electron Microscopy (TEM)

These endpoints have been evaluated at days 14 (4 exposures in 2 days) and 17 (4 exposures followed by 3 days of recovery) after the exposure of the tissues to HW alone or with cleansers, compared to the tissues treated with water with a standard *calcium carbonate* content used as negative control (NC). As reported in the literature, hard water is characterized by a higher concentration of *calcium* and *magnesium carbonates* known to have a harmful effect on the skin barrier integrity leading to dryness increase and potential development of eczematous skin inflammation.

Our results have shown that HW has induced: significant damages to the tight junctions (claudin-1); morphological modification, in particular cell swelling and *stratum corneum* lamellar structure damages; a pro-inflammatory status leading to a severe modification to the barrier structure in the final epidermal differentiation.

The results we have observed in our repeated exposure model agree with the literature findings: histomorphology has shown the loss of specific staining linked to pH modifications, *stratum corneum* lamellar structure was no more observed, there were a significant modification to the tight junctions protein Claudin 1 and a significant expression of TNF-α gene indicating an inflammatory response. The TEER results did not show a damage of hard water alone on the barrier properties due to the thickness increase related to the tissue's swelling, while the damage is observed in the tissue treated with HW + 0.05% SDS highlighting the detrimental effect of the surfactant on the barrier properties.

The study demonstrated that the deleterious effect of hard water on barrier development is worsened by the use of surfactants in daily hygiene routine and the presence of epidermal lipids in this type of toiletries can mitigate the daily early damages providing an efficient recovery of TJs structure and barrier function development.

Hard water itself after 2-day treatment (day 14) modified the granular layer of the epidermis model with the degradation of the lamellar structure (H&E) and modifications in the structural organization of the tight junctions (Claudin immunostaining). More severe effects have been identified at day 17.

However, the main deleterious effects were observed after repeated exposure to 0.05% SDS in HW: increased cytotoxicity and alteration of epidermal thickness, morphology and viability (H&E, CLDN1 expression), reduction of barrier function as observed by TEER values and increase of TNF-α release already at day 14. At day 17, SDS induced severe and deep damage on morphology and barrier function confirming the toxic effect of the detergent. The mild cleanser made of a polymeric surfactant and epidermal lipids counteracted the deleterious effect of the hard water at all levels, suggesting long-term protection of the barrier structure thus reducing the risk of AD development in infants.

In conclusion, the results obtained demonstrated that immature RHE (day 12-14 of in-vitro differentiation) is a good model to mimic the barrier fragility during infancy and to perform a preliminary evaluation of the cleansers' impact on immature skin.

Conflict of interest

The following study was sponsored by Unifarco S.p.A. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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