EFFECT OF DEUTERIUM-DEPLETED WATER ON NORMAL HUMAN SKIN CELLS IN CULTURE AND 3D SKIN EQUIVALENT

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Abbreviations: 3D– tri-dimensional; BM– Basement Membrane; DE– Dermal Equivalent; DEJ– Dermal-epidermal Junction; DS– Dermal Substrate; ECM– Extracellular Matrix; DDW– Deuterium-Depleted Water; UPW– Ultra-pure Water; NHDF– Normal Human Dermal Fibroblast; NHEK – Normal Human Epidermal Keratinocyte; SE– Skin Equivalent.

1. INTRODUCTION

Water is a transparent, tasteless, odorless, and nearly colorless chemical substance that is the main constituent of streams, lakes, and oceans, and the fluids of most living organisms on earth. Deuterium is an isotope of hydrogen, in which the presence of a neutron in the nucleus doubles the mass of the hydrogen atom.[1] In nature, the ratio between deuterium to hydrogen (D/H) is about 1:6600, which means that the natural concentration of D is about 150 ppm (equivalent to a ratio of 0.015%).[2]

Deuterium-depleted water (DDW) or light water, the opposite of heavy water, is microbiologically pure water characterized by a deuterium concentration of 20 – 130 ppm in contrast to a concentration of 140 - 150 ppm in normal water content. Variations occur as depending on the geographical zone and altitude. The amount of deuterium varies from 90 ppm in melted Antarctic ice to 180 ppm in underground water below the Sahara Desert.[1]

It was first reported in 1993 that reduced deuterium concentration in water affects living organisms. [2] And deuterium is now thought to play an important role in the progression of disease and aging.[3][4] A link between aging and deuterium is well established. D_2O concentrations exceeding the natural level resulted in numerous adverse effects: (a) increased viral mutation rates; (b) deuterated enzymes exhibited conformational changes, affecting their active sites; (c) the skin became enriched in deuterium along a temporal aging axis; (d) reduced the lifespan of mice.[5] According to clinical work conducted in Eastern Europe, Japan and Russia, even a seemingly small reduction in deuterium content can influence a number of health parameters.[1]

Although mainstream research focused primarily on the effects of deuterated water in organisms, investigations about DDW is scarce.[6] Recent studies unveiled its important role in the regulation of aging by modulating cell growth and other key biochemical processes.

2. MATERIALS AND METHODS

To conduct such research, normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK) cultured in 3D skin equivalents (SE) were exposed to DDW at different concentrations (50, 132 ppm) and compared to 150 ppm deuterium ultra-pure water (UPW) as a control. The 3D skin equivalent based on a unique collagen-glycosaminoglycan-chitosan porous polymer was cultured in DDW for 33 days. Skin equivalent samples were systematically harvested during the course of culture to evaluate by histology and immunohistology the influence of DDW on skin regeneration.

2.1 In-vitro human SE culture

Primary cultures of NHEK and NHDF were established from healthy human skin obtained from 3 consenting patients undergoing cosmetic surgery, resulting in a surgical discard, in adherence to the Declaration of Helsinki Principles. For the preparation of dermal equivalent, NHDF from adult donors (47 yo, 45 yo, 35 yo) were seeded at a density of 25 x 10^4 cells/cm^2 onto a dermal substrate (DS).[7] For the preparation of skin equivalent, NHEK from donor (16 yo) were seeded on the dermal equivalent (DE) on day 21, at a density of 25 x 10^4 cells/cm^2. After 7 days of submerged culture, SE was raised at the air–liquid interface. DDW was added daily starting from the first medium removal after NHDF seeding (day 1) until the end of the study (day 33).

2.2 Histological and immunohistological analysis

SEs harvested at day 33 of total cell culture were immediately fixed in neutral buffered formalin 4% for 24h or embedded in paraffin or in OCT compound and frozen at -80°C. Paraffin-embedded formalin-fixed samples were then cut into 5 µm sections. After dewaxing and rehydration, sections were stained with Masson's Trichrome for routine histology analysis. Epidermal markers including Ki-67 (DakoCytomation) and filaggrin (Vector Labs), basement membrane components laminin 332 (Abcam), as well as dermal markers collagen I (Abcam), elastin (NOVOTEC) and hyaluronan (CALBIOCHEM) were analysed using histochemistry or immunohistochemistry detection.

2.3 Presentation of the data and statistical analysis

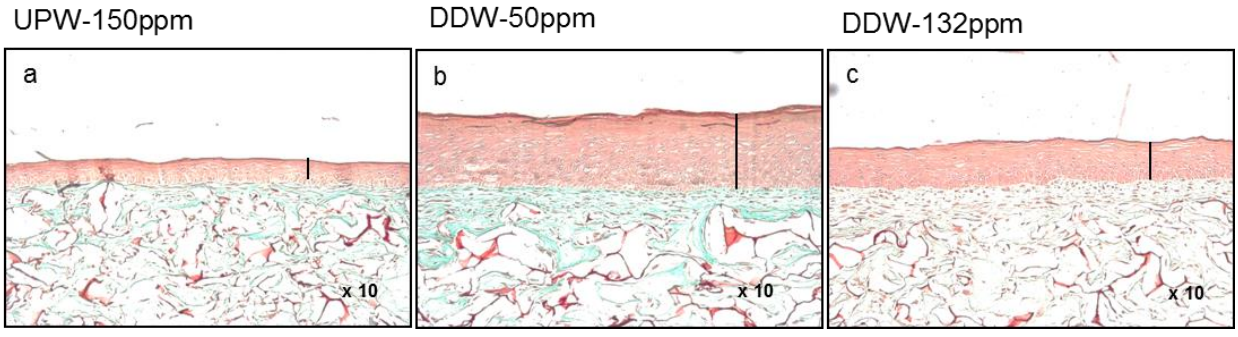
All data are presented as mean values ± standard deviations. Statistical significance in the data was assessed running Student's *t*-test. Each set of data relates to 33-day DDW-treated SE versus to 33-day untreated control. Statistical significant differences are indicated by asterisks as follows: $^{ns}P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

3. RESULTS AND DISCUSSION

Morphological analysis by Masson's trichrome staining showed significant effects of the DDW (50 ppm, 132 ppm) on the reconstruction of epidermal layer compared to the SE untreated control (Fig. 1A).

At the epidermal layer, DDW-treated SE models displayed significant greater thickness of the upper layers (Fig. 1A, b and c) when compared to the untreated control (Fig. 1A, a). The epidermal compartment appeared multi-layered and well-differentiated, whilst the untreated control still showed thin and poorly organized stratum. At the same time, DDW treatment showed a more regular structure in histologic sections, indicating an increased stability of epidermal architecture. Image analysis quantification (Fig 1B) revealed that the reconstructed epidermal compartment in DDW-treated SE samples was significantly thicker ($p<0.001$, paired two tailed Student t-test) than the untreated control (mean±SD; 85.54±7.16 µm, 285.84±18.4 µm and 153.81±13.24 µm for untreated-SE control, DDW-treated SE-50 ppm and DDW-treated SE-132 ppm, respectively).

A



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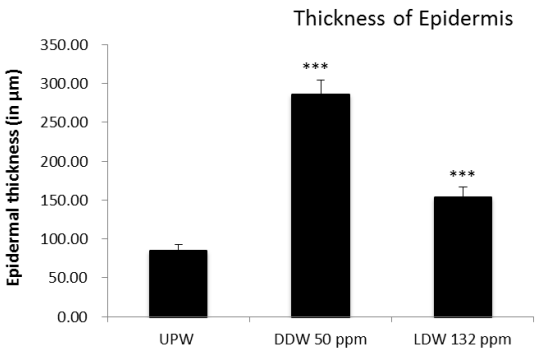


Fig. 1: Influence of DDW supplementation on epidermal compartment in a 3D full-thickness skin healing model engineered with normal human adult skin cells.

A. Histological analysis of SE samples on Day 33.

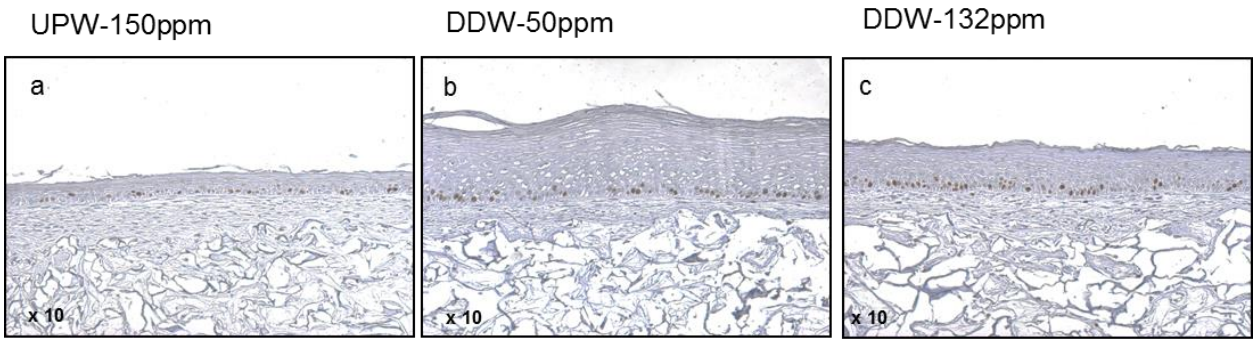
B. Quantification of epidermal thickness by image analysis on Day33.

For each experimental group, SEs were produced in triplicate and experiments were repeated three times. Error bars, SD. ***P< 0.001 compared with the untreated control. Scale bar, 50 µm.

To further investigate the mechanisms underlying the effects of DDW on SE model, we examined the expression of epidermal proliferation and differentiation markers, Ki-67 and filaggrin respectively (Fig. 2).

After 33 days of culture, the number of Ki-67 positive cells significantly increased in the SE treated with the DDW compared to the untreated control (Fig. 2A, a, b, c). Quantitative evaluation of the epidermal proliferation rate by image analysis revealed a significant (p<0.001, paired two tailed Student t-test) increase (Fig. 2B) compared to the untreated control (mean±SD; 34.2±2.1%, 59.4±2.5% and 51.2±1.8% for untreated-SE control, DDW-treated SE-50 ppm and DDW-treated SE-132 ppm, respectively).

A



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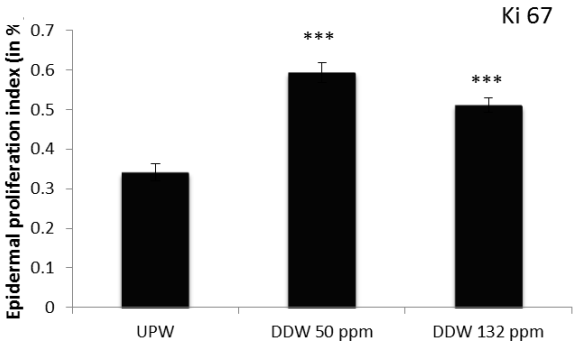


Fig. 2: Influence of DDW on epidermal proliferation marker profile in a 3D full-thickness skin healing model.

A. Optical microscopy observation of epidermal proliferation Ki-67 immnunoperoxidase staining.

B. Image analysis quantification of epidermal proliferation index.

For each cell culture condition and analysis, SEs were produced in triplicate and experiments were repeated three times. Error bars, SD. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the untreated control. Scale bar, 50 µm.

About the epidermal differentiation, the terminal stage was evidenced by expression of filaggrin (Fig. 3A, a, b and c). Obviously, DDW-treated SE samples revealed a positive staining of the filaggrin (Fig. 3A, b, c), while only sporadic clusters were detected in the 33-day SE untreated control (Fig. 3A), suggesting an enhancement of late differentiation process. Image analysis (Fig. 3B) showed a significant (p<0.01/0.05, paired two tailed Student t-test) increase of filaggrin expression (mean±SD; 4.48±0.53 pixel^2/µm, 24.90±0.82 pixel^2/µm and 10.70±0.66 pixel^2/µm for untreated-SE control, DDW-treated SE-50 ppm and DDW-treated SE-132 ppm, respectively). The results indicated that DDW can

promote the maturity of the SE constructs.

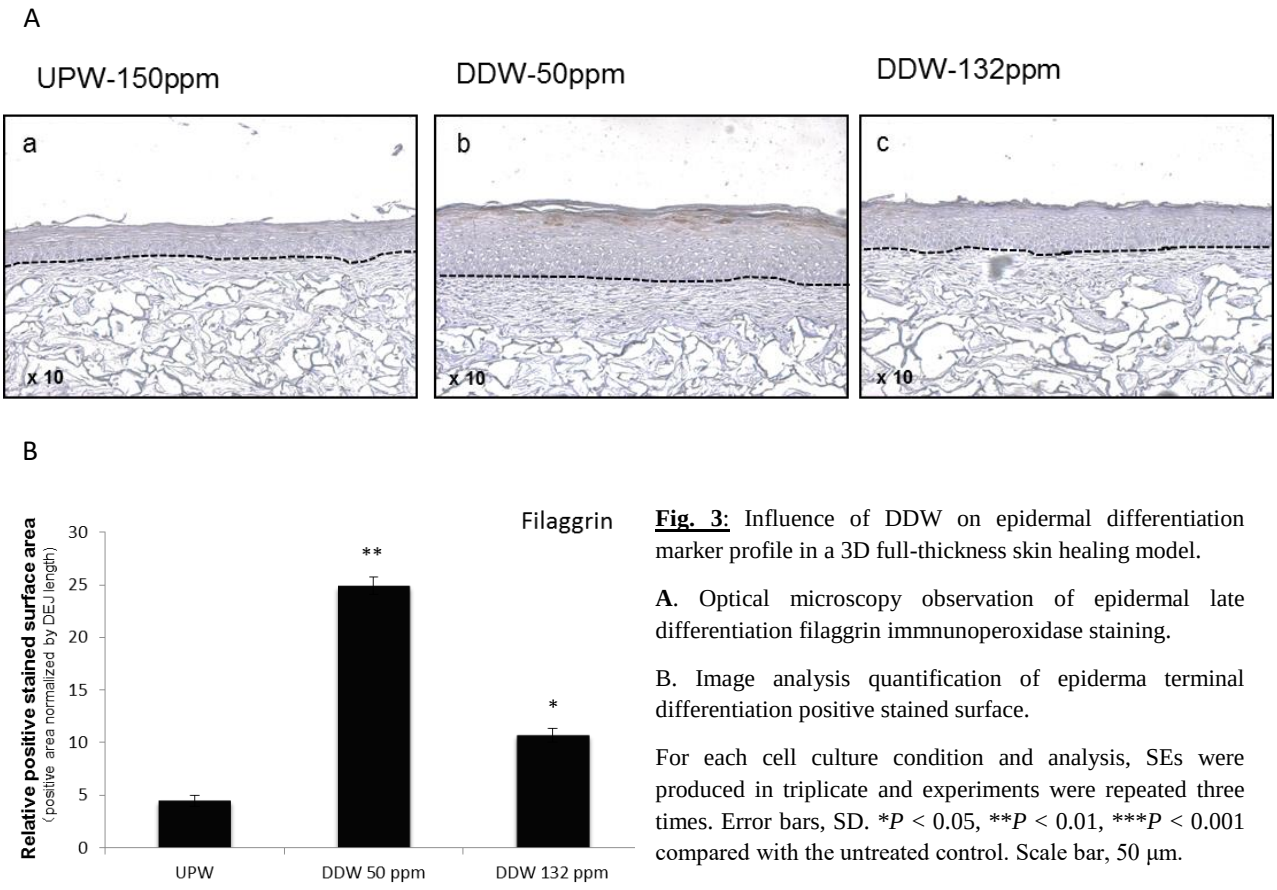


Fig. 3: Influence of DDW on epidermal differentiation marker profile in a 3D full-thickness skin healing model.

A. Optical microscopy observation of epidermal late differentiation filaggrin immnunoperoxidase staining.

B. Image analysis quantification of epiderma terminal differentiation positive stained surface.

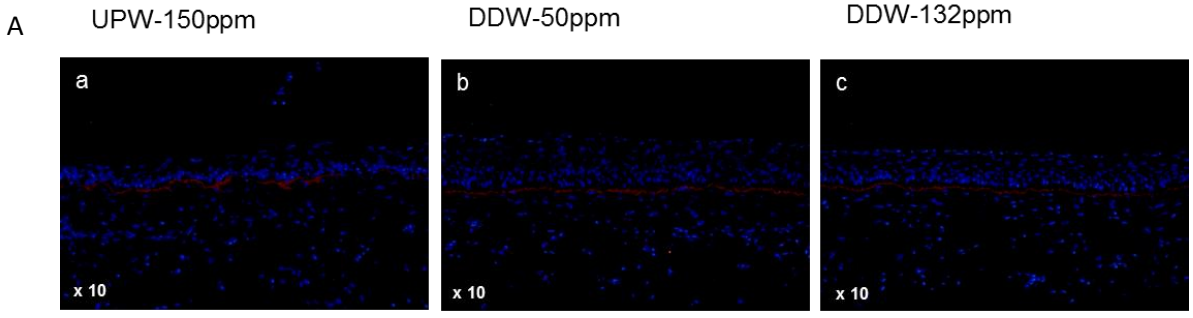
For each cell culture condition and analysis, SEs were produced in triplicate and experiments were repeated three times. Error bars, SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the untreated control. Scale bar, 50 μm.

In a word, the results showed that greater epidermal thickness was the result of increased keratinocyte proliferation, together with complete terminal differentiation, suggesting that DDW accelerates epidermal regeneration process in a complete homeostasis.

At the DEJ level, dermal fibroblasts and epidermal keratinocytes interact together to form an interlinked BM of ECM, which forms as a concerted action of both keratinocytes and fibroblasts.

The expression of crucial BM proteins involved in skin healing process and epidermal integrity such as laminin 332 was analyzed by immunostaining (Fig. 4). After 33 days of culture, laminin 332 was weakly expressed in the untreated control (Fig. 4A, a). In contrast, the expression of this protein was enhanced in DDW 50 ppm-treated SE constructs (Fig. 4A, b), indicating a more advanced tissue maturation.

These immunohistochemical observations were further confirmed by automated quantification, and indicated a statistically significant ($p<0.05$/ns, paired two tailed Student *t*-test) increase in the expression of laminin 332 involved in the morphogenesis of the DEJ (Fig. 4B). (mean±SD; 9.66±3.59 pixel^2/μm, 25.80±0.27 pixel^2/μm and 22.07±1.67 pixel^2/μm for untreated-SE control, DDW-treated SE-50 ppm and DDW-treated SE-132 ppm, respectively).



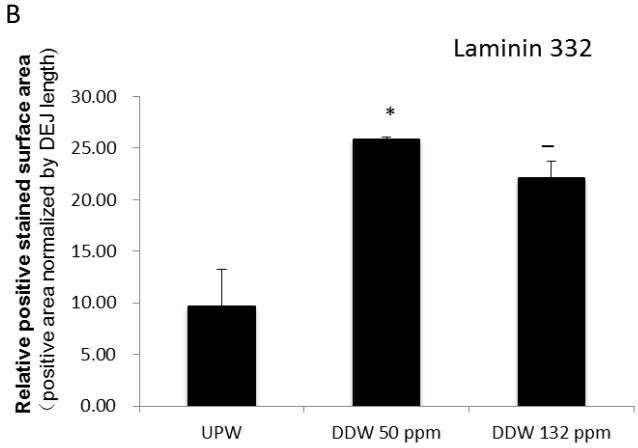


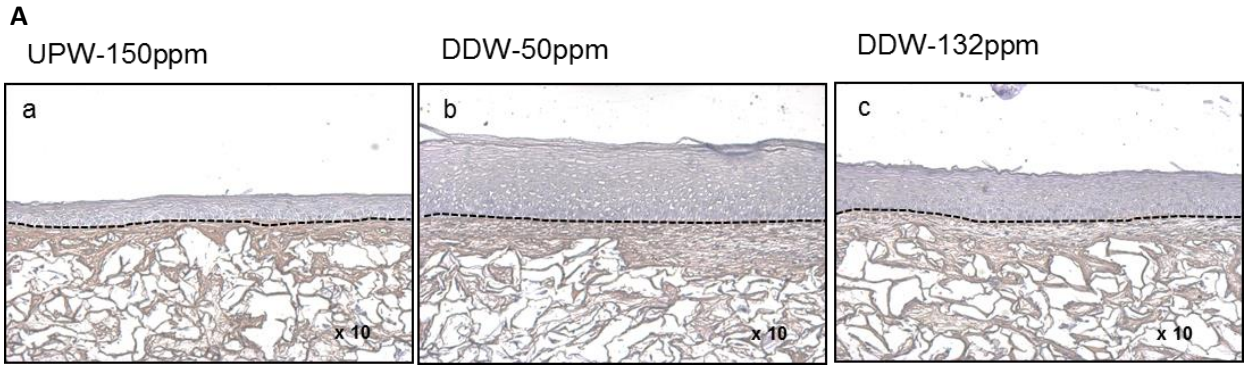
Fig 4: Influence of DDW on epidermal basement membrane morphogenesis and assembly in a 3D full-thickness skin healing model cultured 33 days.

A. Optical microscopy observation of laminin 332 immunofluorescence staining.

B. Image analysis quantification of laminin 332 positive stained surface.

For each cell culture condition and analysis, SEs were produced in triplicate and experiments were repeated three times. Error bars, SD. $^{ns}P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared with the untreated control. Scale bar, 50 µm.

To further study the effects of DDW on dermal maturation, we investigated the expression of type I collagen, hyaluronan and elastin, three important regulators in maintaining the ECM and tissue repair. Immunostaining analysis of type I collagen revealed an increase of its expression in the DDW 50 ppm-treated SE. The stimulation of this dermal structural protein suggested an acceleration of dermal maturation by the DDW (Fig. 5). (mean±SD; 0.55±0.02 pixel^2/µm, 0.66±0.04 pixel^2/µm and 0.58±0.02 pixel^2/µm for untreated-SE control, DDW-treated SE-50 ppm and DDW-treated SE-132 ppm, respectively).



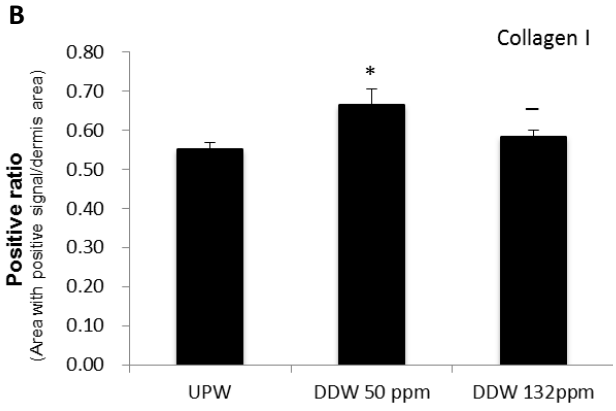


Fig 5: Influence of DDW on dermal components expression involved in ECM formation and remodeling in a 3D full-thickness skin healing model cultured 33 days.

A. Histochemical analysis of type I collagen in DDW-treated SE.

B. Image analysis quantification of dermal components positive stained surface.

For each cell culture condition and analysis, SEs were produced in triplicate and experiments were repeated three times. Error bars, SD. $^{ns}P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the untreated control. Scale bar, 50 µm.

Hyaluronan is the main component of the dermal ECM and is also present in the epidermis and has a moisturizing effect on the skin. Staining for hyaluronan, using a biotinylated hyaluronic acid-binding peptide, was also positive in the whole DE of the untreated control with a slightly higher intensity in the upper part of the dermis (Fig. 6). DDW-treated SE showed an obviously increase of the stained surface area localized in the epidermis and dermis. (mean±SD; 0.30±0.003 pixel^2/µm, 0.61±0.003 pixel^2/µm and 0.50±0.008 pixel^2/µm for untreated-SE control, DDW-treated SE-50 ppm and DDW-treated SE-132 ppm, respectively).

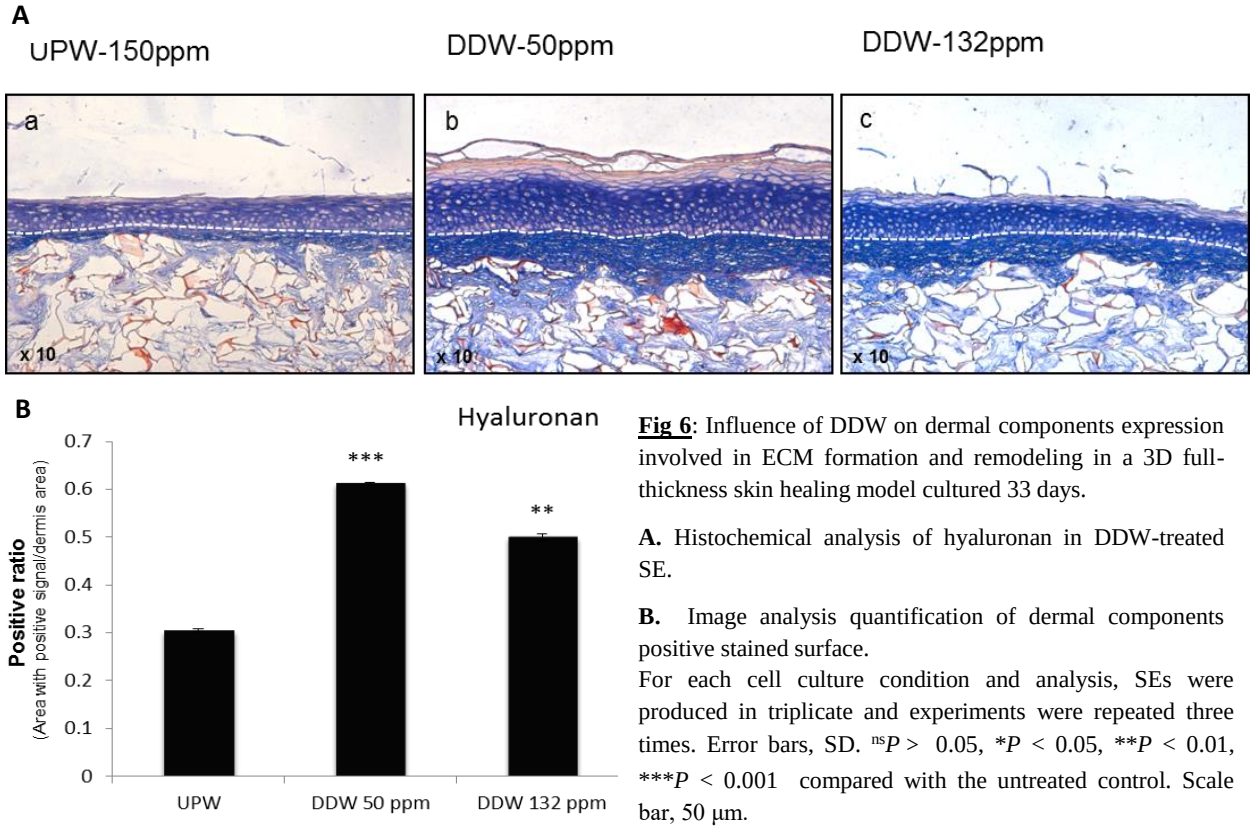


Fig 6: Influence of DDW on dermal components expression involved in ECM formation and remodeling in a 3D full-thickness skin healing model cultured 33 days.

A. Histochemical analysis of hyaluronan in DDW-treated SE.

B. Image analysis quantification of dermal components positive stained surface.

For each cell culture condition and analysis, SEs were produced in triplicate and experiments were repeated three times. Error bars, SD. $^{ns}P >$ 0.05, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the untreated control. Scale bar, 50 µm.

As shown in Fig. 7, representative imaging revealed a regular and consistent deposition of elastin through the DE in the untreated control. In DDW 50 ppm-treated SE, the synthesis of this matrix protein was highly enhanced suggesting the promotion of ECM elasticity and stability. (mean±SD; 0.02±0.006 pixel^2/µm, 0.06±0.015 pixel^2/µm and 0.04±0.017 pixel^2/µm for untreated-SE control, DDW-treated SE-50 ppm and DDW-treated SE-132 ppm, respectively).

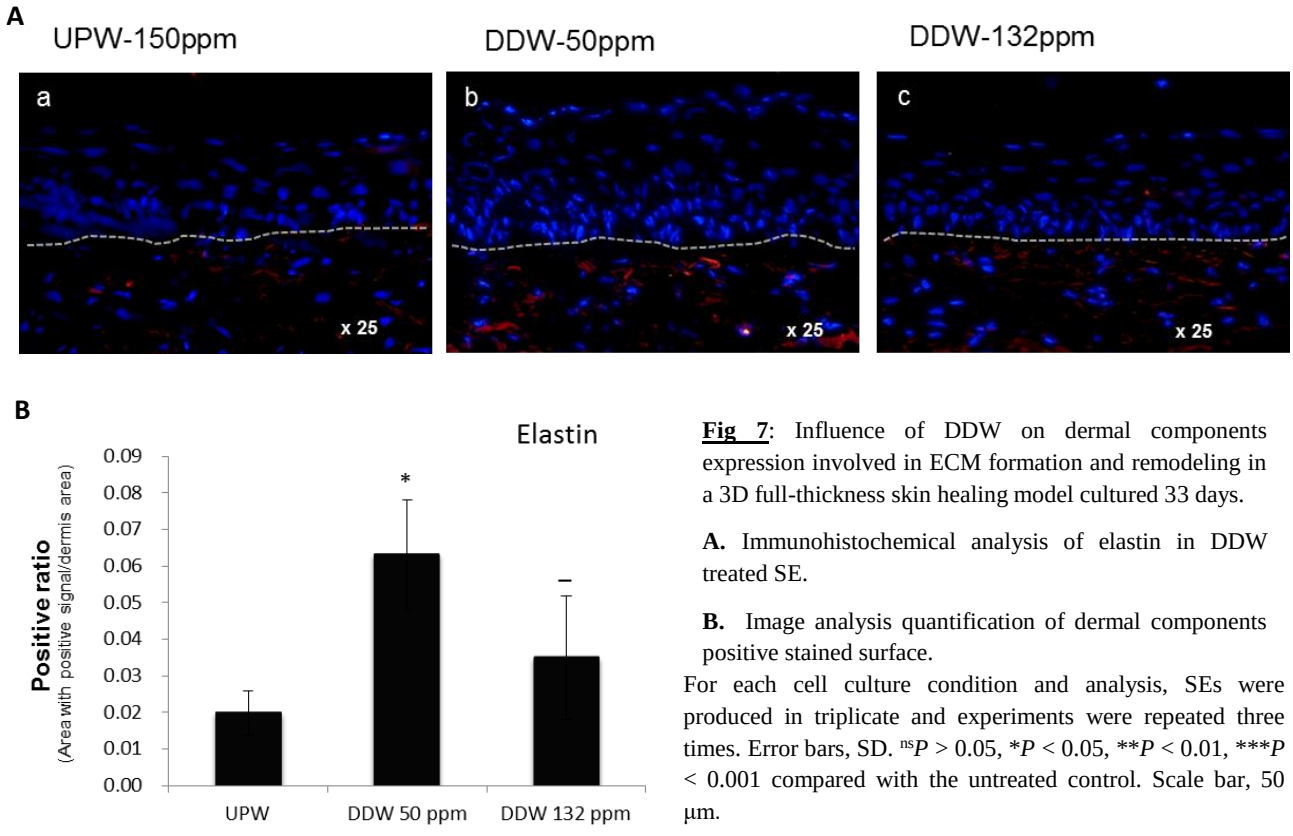


Fig 7: Influence of DDW on dermal components expression involved in ECM formation and remodeling in a 3D full-thickness skin healing model cultured 33 days.

A. Immunohistochemical analysis of elastin in DDW treated SE.

B. Image analysis quantification of dermal components positive stained surface.

For each cell culture condition and analysis, SEs were produced in triplicate and experiments were repeated three times. Error bars, SD. $^{ns}P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the untreated control. Scale bar, 50 µm.

These immunohistochemical and histochemical observations demonstrated a significant increase of elastin, hyaluronan and type I collagen deposition in the DDW 50 ppm-treated SE compared to the untreated control.

In this study, we used a 3D full-thickness SE model to investigate the biological effects of daily DDW treatments on skin regeneration and healing. According to epidermal differentiation process, ECM maturation and DEJ morphogenesis during the culture showed that DDW (50-132 ppm) has significant effects on the reconstruction of both epidermal and dermal layers.

These results support for the first time the importance of deuterium in skin healing process, and provide an insight into DDW utilization in the formulation of dermatological products. The results also show the uses of the full-thickness skin equivalent engineered with a collagen-glycosaminoglycan-chitosan scaffold as a model for evaluation of parameters involved in skin regeneration.

4. CONCLUSION

The results showed that DDW treatment significantly accelerated the reconstruction of both epidermal and dermal compartments in the 3D skin equivalent. The epidermis appeared thicker and well-differentiated, however the untreated control (UPW) showed a thin and poorly organized structure. At the same time, DDW stimulated the synthesis of the ECM at the dermal level. Histological and immunohistological analysis confirmed the accelerated epidermal and dermal maturation and showed a significant increase of Ki-67 proliferation and filaggrin differentiation epidermal markers and a significant stimulation of extracellular matrix protein deposition such as collagen I, hyaluronan and elastin. Concomitantly, DDW induced a higher expression of basement membrane proteins associated with re-epithelisation such as laminin 332.

These results demonstrate the influence of DDW on skin cells proliferation, differentiation, and extracellular matrix synthesis in 3D full-thickness skin equivalent model, and further support the importance of DDW in cosmetic applications.

REFERENCES

[1] M. J. Pangman, Effects of deuterium-depleted water on cell membrane integrity and overall health, as measured by heart rate variability, bioelectrical impedance analysis and galvanic skin response. Water Conference 2016.

[2] G. Somlyai, G. Jancsó, G. Jákli, M. Molnár, G. Laskay, L. Z. Fehér, L. G. Puskás, Naturally occurring deuterium is essential for the normal growth rate of cells. Federation of European Biochemical Societies 1993, 317, 1-4.

[3] T. R. Griffiths, The Possible Roles of Deuterium in the Initiation and Propagation of Aging and Other Biological Mechanisms and Processes. Proceedings of the Second International Conference on Stable isotopes. 1975, 20-23.

[4] G. Somlyai, Defeating Cancer: The Biological Effects of Deuterium Depletion, 1st Books Library, 2001.

[5] D. S. Ávila, G. Somlyai, I. Somlyai, M. Aschner, Anti-aging effects of deuterium depletion on Mn-induced toxicity in a C. elegans model. Toxicology Letters 2012, 211, 319-324.

[6] F. S.Cong, Y. R.Zhang, H. C. Sheng, Z. H. Ao, S. Y. Zhang, J. Y. Wang, Deuterium-depleted water inhibits human lung carcinoma cell growth by apoptosis. Experimental and Therapeutic Medicine 2010, 1, 277-283.

[7] F. Duplan-Perrat, O. Damour, C. Montrocher, S. Peyrol, G. Grenier, M. P. Jacob, F. Braye, Keratinocytes influence the maturation and organization of the elastin network in a skin equivalent. J. Invest. Dermatol. 2000, 114, 365-370.