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New ex vivo model in wound care highlights a natural skin regenerating booster

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ABSTRACT (250 WORDS)

Wound healing is a complex mechanism which is implemented by the perfect coordination of 4 phases: hemostasis, inflammation, proliferation, and remodeling. Excessive and prolonged inflammation promotes wound healing delay and excessive scaring. Thus, the switch between proinflammatory phase and skin reparative phase (proliferative and remodeling phases) is crucial. We propose the use of punched skin explants as a skin wound healing model to mimic this delicate transition and thus screen effective products that may accelerate this process. In a first step, we investigated the natural repairing kinetic of this injury model by analyzing epidermis and dermis markers at mRNA and protein levels. In a second step, a promising cream was applied on punched skin explants every two days for 14 days to evaluate its regenerating properties.

Seven days after the wound, the model exhibited the hallmarks of the inflammation phase by repressing the gene expression of remodeling and differentiation markers. Fourteen days later, our model displayed characteristics of the early proliferative phase. We then showed that the cream, composed of a synergic combination of vegetable oils, a bud extract, and young shoots extracts, promotes entry into the proliferative phase from seven days after the wound; in contrast, untreated skin appeared to achieve these characteristics fourteen days after the wound.

In conclusion this in vitro experimental wound healing model offers a novel approach to highlight product that can accelerate the wound healing process. The model suggested that the promising cream can save at least 7 days on the wound healing process.

KEYWORDS

Wound healing – skin regenerating – skin model - cream-

INTRODUCTION

The wound healing process is a natural skill of the skin that enables the repair of skin lesions after different kinds of physical or chemical injuries. This complex process needs to be coordinated between the different cellular and molecular components involved in wound healing. It is composed of 4 phases controlled by various signaling molecules, hormones, proteases, and cytokines: homeostasis, inflammation, cell proliferation and remodeling (*1*).

The wound healing process starts immediately after the injury with the homeostasis phase, to stop the bleeding by creating a fibrin clot. Structural proteins and proteases

(fibrinogen, fibronectin, vitronectin, thrombospondin…) are released from the blood capillaries to create a molecular network that allows the blood to coagulate (*2*). Meanwhile cytokines, including interleukin-1 (IL-1), platelet-derived growth factor (PDGF), are secreted by surrounding cells and platelets as a first signal to alert other cells from the injury, attract immune cells (macrophages) toward the wound, and start the inflammation phase (*3*). This inflammation phase protects the wound from infections due to invading pathogens which could penetrate because of the break of the skin epidermal barrier. Macrophages infiltrate to the injured zone to protect the clot and produce cytokines and hormones (Tumor necrosis factor α (TNFα), IL-6, IL-1β, vascular endothelial growth factor (VEGF) etc.) in response to the inflammatory stimuli (*4*–*7*). Inflammatory cytokines, such as IL-6, control the switch between inflammatory and proliferation phases, by stimulating the production of protease to locally destroy the surrounded extracellular matrix and to allow the cells to proliferate and to migrate into the wound. This proliferation phase fills the wound by a re-epithelization process, repairing the vascular network and reconstructing the granulation tissue. Key factors, such as proliferative cell nuclear activator (PCNA), epidermal growth factor (EGF), collagen III, α smooth muscle actin and proteases are stimulated to manage this phase (*3*, *8*, *9*). Eventually, after several weeks, the remodeling phase improves wound contraction and restores the normal structure and function of the epidermis and the dermis, managed by differentiation factors such as transforming growth factor β, collagen I, filaggrin, keratins loricrin, etc. (*10*–*12*).

The quality of the wound healing process depends on the duration of the inflammatory phase and on the crucial switch to the proliferative phase, under the control of the balance between pro- and anti-inflammatory cytokines. Several externals and internal factors can modulate this balance by enhancing the production of pro-inflammatory cytokines (IL1, IL6 etc.) and/or reducing the expression of anti-inflammatory factors (IL10) (age, diabetes, infections, skin diseases, exposome). In consequence, wound healing is delayed, resulting in chronic wounds and excessive scarring (*1*, *13*, *14*). Indeed, 80 % of chronic wounds are associated with venous or arterial insufficiency, decubitus, or diabetes (*15*).

Natural solutions could help the healing process as alternative to therapies for faster healing by improving the quality and the duration of the inflammation phase. A lot of natural compounds display pharmacological activities involved in the wound healing process such as anti-inflammatory, antimicrobial, antioxidant, immunomodulatory, antiviral and enhanced myofibroblast activities (*16*). Natural compounds present in plant aqueous extracts, such as quercetin, can accelerate the wound healing process by modulating the antioxidant system of wound, cytokines, growth factors and cells involved this process (*17*). Vegetable oils are also interesting in this context due to their semi-occlusive properties, protecting the wound from pathogens and allergens, and their emollient function, participating in the restoration of the epidermal barrier(*18*). Natural components can be used in dermo-cosmetic balms to create a natural solution to improve and accelerate the wound healing process.

In this context, the need of wound healing model that mimics the delicate transition between inflammatory and the proliferative phase is crucial in order to screen natural ingredients as well as dermo-cosmetic balms and creams. In this study, we implemented a wound healing model obtained from human skin explant by a double excisional biopsy punch (10mm and 2 mm) and characterized the natural repairing kinetics of the injury by analyzing epidermis and dermis markers at mRNA and protein levels. A vitality cream composed with active

compounds from bud extract, plants oils and hydrolats was then screened to assess its regenerative properties on this new model.

METHODS

Skin

Human skin explants were obtained from a 55 years-old Caucasian female donor undergoing abdominoplasty surgery. The donor gave her informed consent, and the skin samples were received and immediately processed. The adipose tissues were removed and 10mm biopsy punches were performed, followed by a secondary 2mm punch at the center. The explants were then placed into cell culture inserts and loaded into a multi-well culture plate (BD Falcon). They were fixed into a solid nourishing fibrinogen matrix.

Cream description

The vitality cream, (Biovive 5664-1.02 lot 1904288), was provided by Laboratoires Gilbert. The products were stored at +4°C, according to their instructions. The cream was applied into the wound and around the wound on the explants for topical application.

Culture and treatments

The culture medium dedicated to the skin explants was renewed every other day. Skin biopsies were grown and processed separately. The different conditions, each performed in triplicate, were:

- Untreated control (NTW) for 0, 7 or 14 days.
- Sample treated with Biovive (Biovive Wound) for 7 and 14 days.

Excess active ingredient from the previous day was removed with a cotton swab. The Vitality cream was deposited in the wound using a 1ml syringe without needle. A drop of PBS +/+ was put in the wound (about 10µl/hole) for the wound control.

On days 7 or 14, each punch biopsy was cut into 2 parts. One part was used for RNA extraction and PCR analysis, while the other was fixed in OCT for histological assays.

Gene screening

Total RNAs from samples were homogenized in Trizol reagent (Thermo Fisher Scientific: TRIzol Plus RNA purification kit). The quality controls were performed using Agilent RNA nano kit with Analysis Agilent 2100 bioanalyzer. The total RNA quantitation was performed using a Nanodrop spectrophotometer (Appendix II).

For target quantitation, RNA was reverse transcribed into cDNA with Superscript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR was performed with a Platinum Quantitative PCR SuperMix-UDG Kit (Invitrogen) using the CFX system (Bio-Rad). PCR primers were computationally designed and bought from our knowledgeable suppliers. PCR were performed in duplicates for each biological sample. The results were normalized against GAPDH housekeeping gene expression as an endogenous control.

Skin punch biopsy processing before immunohistochemistry and h&e

Molds were filled with an Optimum Cutting Temperature medium (Thermo Fisher, Shandon Cryomatrix, 6769006) for cryopreservation and the samples were immersed inside with the required orientation, and then frozen on a dry ice/96% Ethanol bath. Once the freezing temperature was achieved, the samples were kept at -80°C before sectioning.

Each biopsy was then placed on a sample block within a cryostat cabinet (Leica CM3050 S) set at a -25ºC. The frozen block (containing the biopsy) was sectioned at a thickness of 8 µm and sections were mounted on polylysine superfrost slides (Thermo Fisher Superfrost Plus). The slides were stored at -80°C prior to staining.

Hematoxylin & eosin staining

Slides were thawed for 5 minutes, then fixed in formalin for 10 minutes. After 2 rinses (PBS then tap water) the slides were incubated with Hematoxylin solution (Mayer's Hematoxylin Solution; Sigma MHS16) for 5 min. After rinsing twice with tap water, the slides were successively incubated with 70% ethanol, 96% ethanol, and 0.1% eosin (1% RAL Aqueous Eosin, Ref. 312740-250, diluted 1:10 in water) for 30 seconds each.

After rinsing twice with tap water, the slides were dried with 96% ethanol twice for 30 seconds and then with 100% ethanol twice for 30 seconds. Finally, the slides were incubated 3 times with Xylene for 1 minute and stored in Eukitt mounting medium (Sigma Aldrich, 03989). Images were then taken with an upright microscope (Zeiss, Axio Imager Z1, ApoTome, Zen2 blue edition software) equipped with a brightfield channel and a color camera.

MMP3 or PCNA immunostaining

Slides were thawed for 5 minutes and then fixed in Formalin for 15 minutes for staining of PCNA and MMP3. They were incubated in TBS-T 5% BSA at room temperature for 1 hour to prevent nonspecific binding of the primary antibody. Next, the slides were incubated overnight at 4°C with specific primary antibody of PCNA (D3H8P XP (R) Rabbits mAb #13110) or MMP3 (AF513 biotechne RetD Systems).

Following this, the slides were incubated with secondary antibody anti-rabbit Cyanine 5 (Jackson Lab) for staining of PCNA or secondary antibody anti-goat Alexa Fluor 647 (Invitrogen) for 1h at room temperature in the dark for staining of MMP3. Finally, the slides were incubated with Hoechst® 33342 1/5000e for 10 minutes and preserved in mounting medium Fluoromount-G (Thermo Fisher Scientific, 00-4958-02). Each step was followed by 3 washings with TBS-T for 5 minutes. The images were then taken with an epifluorescence microscope (Zeiss, Axio Imager Z1, ApoTome, Zen2 blue edition software) equipped with a Cyanine 5 channel and analyzed with a homemade algorithm.

Quantification strategy and statistical tests for immunostaining

To study the effect of the cream, an algorithm was designed to perform to quantify the expression PCNA and MMP3 on skin sections. The first step consisted of selecting the epidermis and/or dermis, region of expression of the markers. The algorithm used was then able to measure the Cy-5 mean intensity fluorescence in the cytoplasm of the cells inside the selected region for each image.

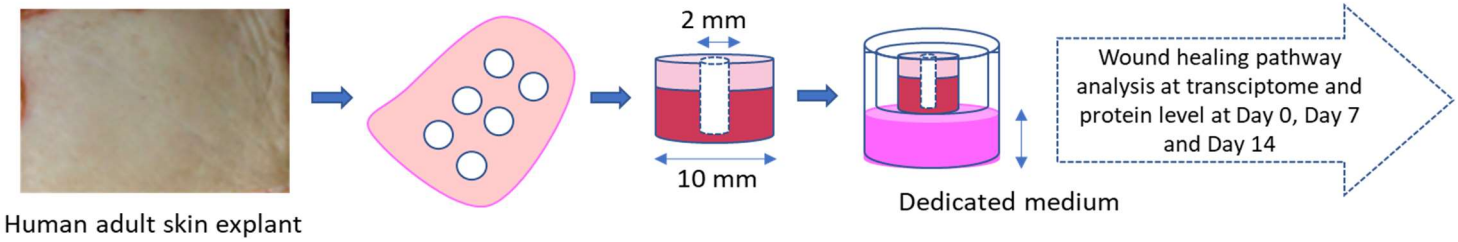


Figure 1 ASEM methods.

Skin explants are provided by abdominoplasty of consenting adults. Ten millimeters punch biopsies were removed. A wound of 2 mm in the center of the 10 mm punch biopsy were performed. Punches were placed into a 12-well tissue culture dish with well chamber inserts, embedded in fibrinogen matrix and exposed to air–liquid interface with dermis-side down in a dedicated medium at 37°C, 5% CO_2. All punches models were and collected on day 0, 7 and 14 after punch dissection.

RESULTS

Wound healing skin explant model description

In this study, we developed a new model of wound healing based on human skin explant. We performed a double excisional skin wound in human skin explant using 10 mm and 2 mm punches to obtain a ring-shaped skin explant. This annular skin explant model (ASEM) is placed into a 12-well tissue culture dish with dedicated medium. Figure 1 schematizes the method to obtain the ASEM.

Characterization of ASEM that mimics wound healing mechanisms

To characterize ASEM over time, we firstly evaluated by quantitative PCR the gene expression regulation of 12 genes involved in inflammation, proliferation, and remodeling wound healing phases at days 0, 7 and 14 after ASEM production. GAPDH housekeeping gene was used for normalization. The graphs presented in figure 2 showed the quantitative expression of the 12 genes at days 0, 7, 14. From Day 0 to Day 7, IL6 and MMP3 genes increased in expression while EGFR, TGFB, MMP19, PCNA, COL3A1, ACTA2, COL1A1, LAMA5, FLG, LOR drastically decreased. From day 7 to day 14, the induction of IL6 and the repression of EGFR, MMP19, TGFB, ACTA2, LAMA5, LOR and FLG was maintained or accentuated while MMP3 induction and repression of PCNA, COL3A1 and COL1A1 gene repression are counterbalanced. Taken together, these results suggested that the period from day 7 to day 14 marked a switch in the wound healing mechanism. To investigate the effect of a new cream, ASEM were treated by the cream during 7 or 14 days after ASEM production. Treatment with the Vitality cream had no effect on FLG, LAMA5, EGFR gene regulation. However, from day 0 to day 7, our results showed that this treatment accelerated MMP19 repression and reduced MMP3 induction. From day 7 to day 14 the cream treatment inhibited IL6, PCNA, COL31 and COL1A1 induction and increased the expression of ACTA2 and LOR genes. Interestingly, the cream had the tendency to induce the TGFβ2 expression. This up-regulation is not statistical due to the high variability of the data.

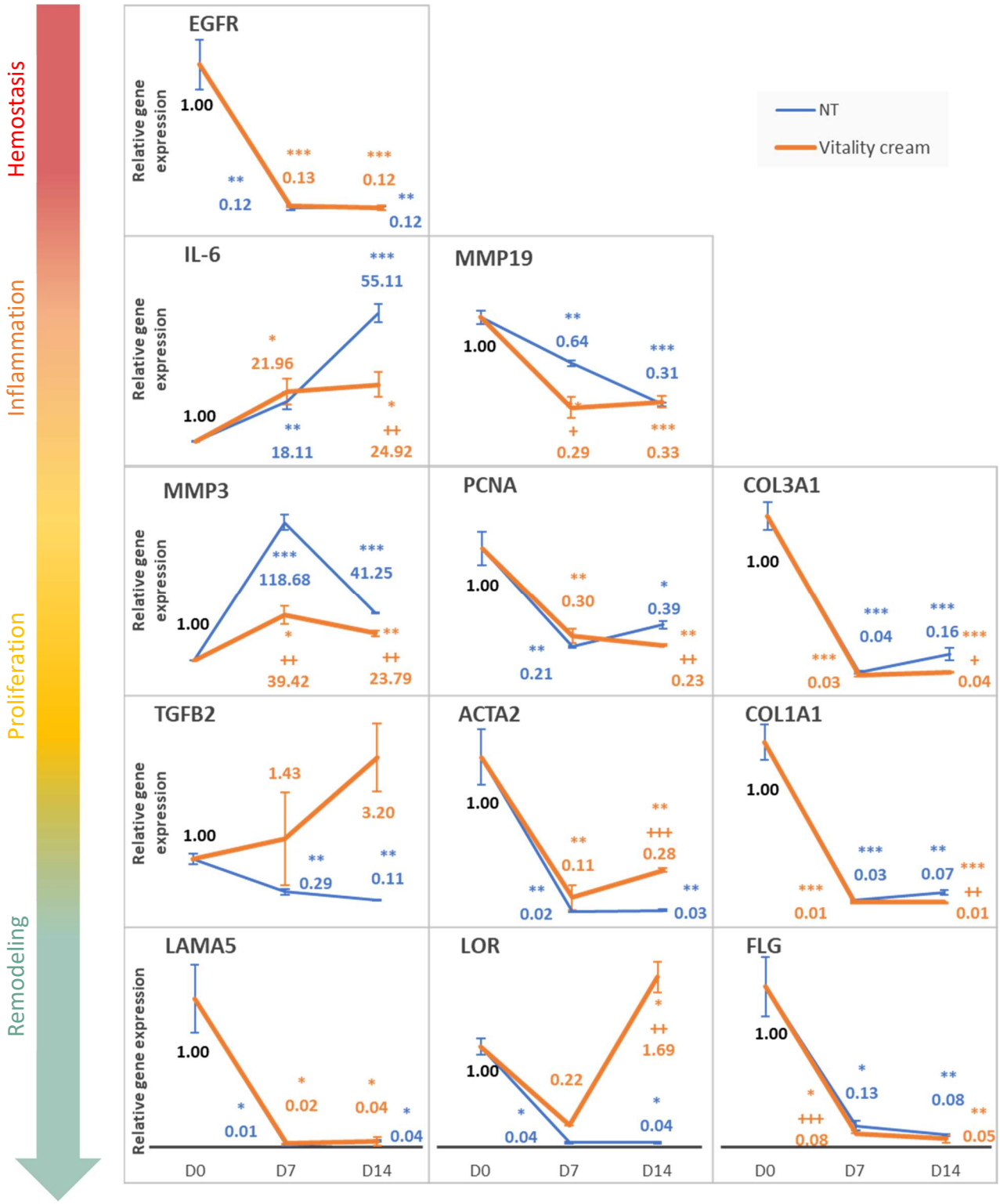
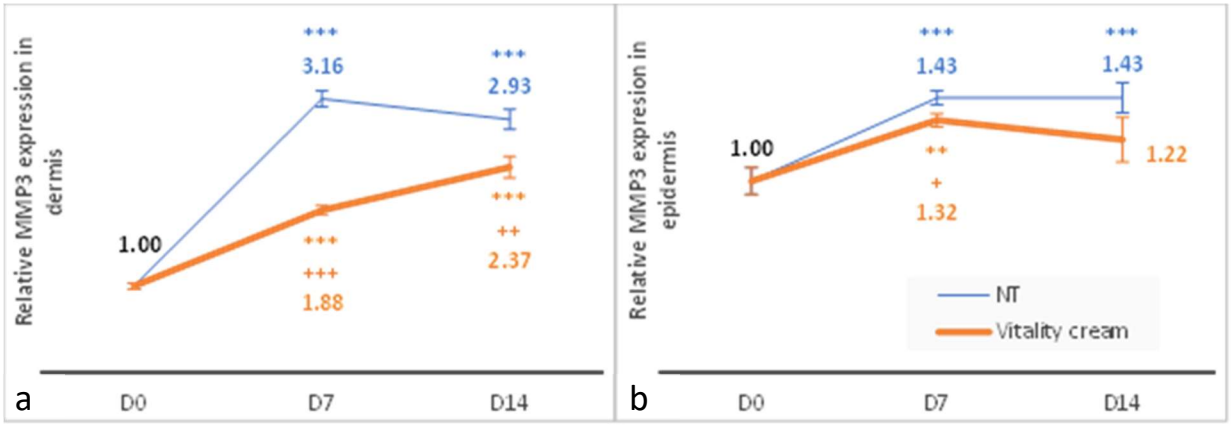


Figure 2 Inflammation/Proliferation switch after 7 days during wound healing.

Wounded explants were treated with Biovive or untreated (NT). RNAs were extracted after 7 or 14 days after the wound and treatment and were compared to the control at J0 (wounded, untreated). Each experiment was done in triplicate. Gene expression levels were measured by RT-qPCR, and expression ratio levels were calculated for each condition against the control. The results were normalized to endogenous control GAPDH expression. The error bars represent the SEM. Statistical analysis was performed using T-tests against the untreated condition: (* p-value<0.05, ** p-value<0.005, * p-value<0.001) against J0 and (+ p-value) against the same day untreated condition.

However, a previous study on explant had proved that the cream had the ability to statistically induce the expression of TGFβ2 (data not shown).



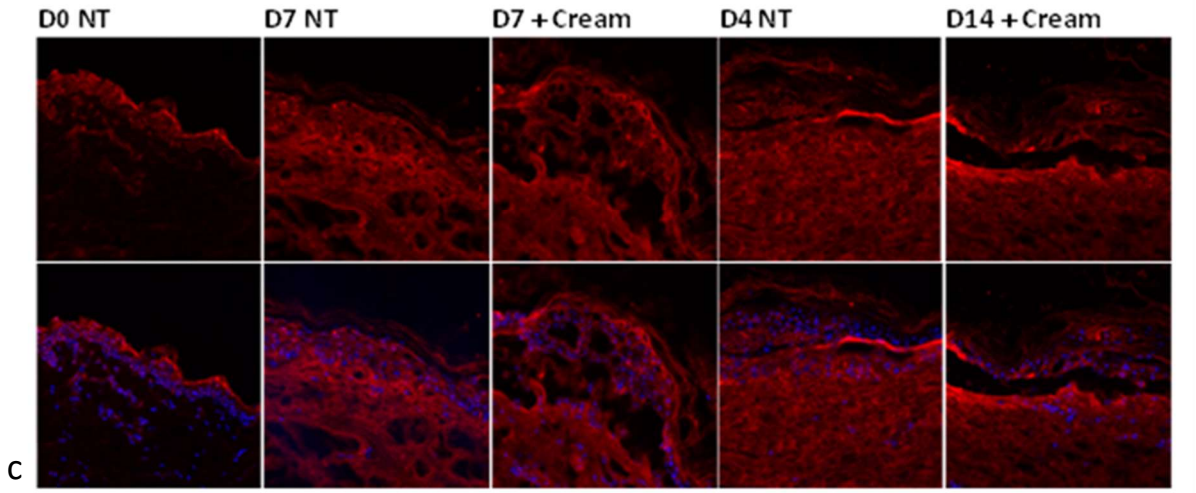


Figure 3 MMP3 IHC labelling.

Wounded explants were treated with Biovive or untreated (NT). The explants where sectioned and stained after 7 or 14 days and compared to the control at J0. The mean fluorescence intensity was measured inside the dermis (a) or the epidermis (b). Expression levels were computed relatively to the control. The error bars represent the SEM. Statistical analysis was performed using T-tests against the untreated condition: (* p-value<0.05, ** p-value<0.005, *** p-value<0.001) against J0 and (+ p-value) against the same day untreated condition.

Examples of pictures of the stained sections for each condition are shown in (c). In the first row only the labelling of MMP3 is shown in red. In the second row nuclei are also labelled in blue.

MMP3 protein peaks at day 7 in the dermis and the epidermis of our ASEM

In our ASEM, MMP3 gene expression peaked at day 7 suggesting a switch from day 7 to day 14 in the wound healing process. To corroborate this regulation at protein level, we performed Immunohistochemistry staining of MMP3. Quantification of MMP3 positive areas in the dermis and the epidermis showed that MMP3 protein increased from day 0 to day 7 in the dermis and the epidermis (figure 3). This induction had the tendency to decrease at day 14. The MMP3 protein expression peak at day 7 was inhibited by the cream treatment. Our protein expression data are consistent with our mRNA expression.

Key marker of proliferative phase, PCNA was regulated in the epidermis

While MMP3 was transiently peaked at day 7, several genes including PCNA were also transiently downregulated at day 7. To support this regulation at protein level, we performed Immunohistochemistry staining of PCNA protein (figure 4). Quantification of PCNA positive areas in the epidermis of our ASEM revealed that PCNA protein expression was transiently down

regulated from day 0 to day 7 and the expression of PCNA seemed to increase from day 7 to day 14. The cream treatment maintained the PCNA repression between day 7 and day 14.

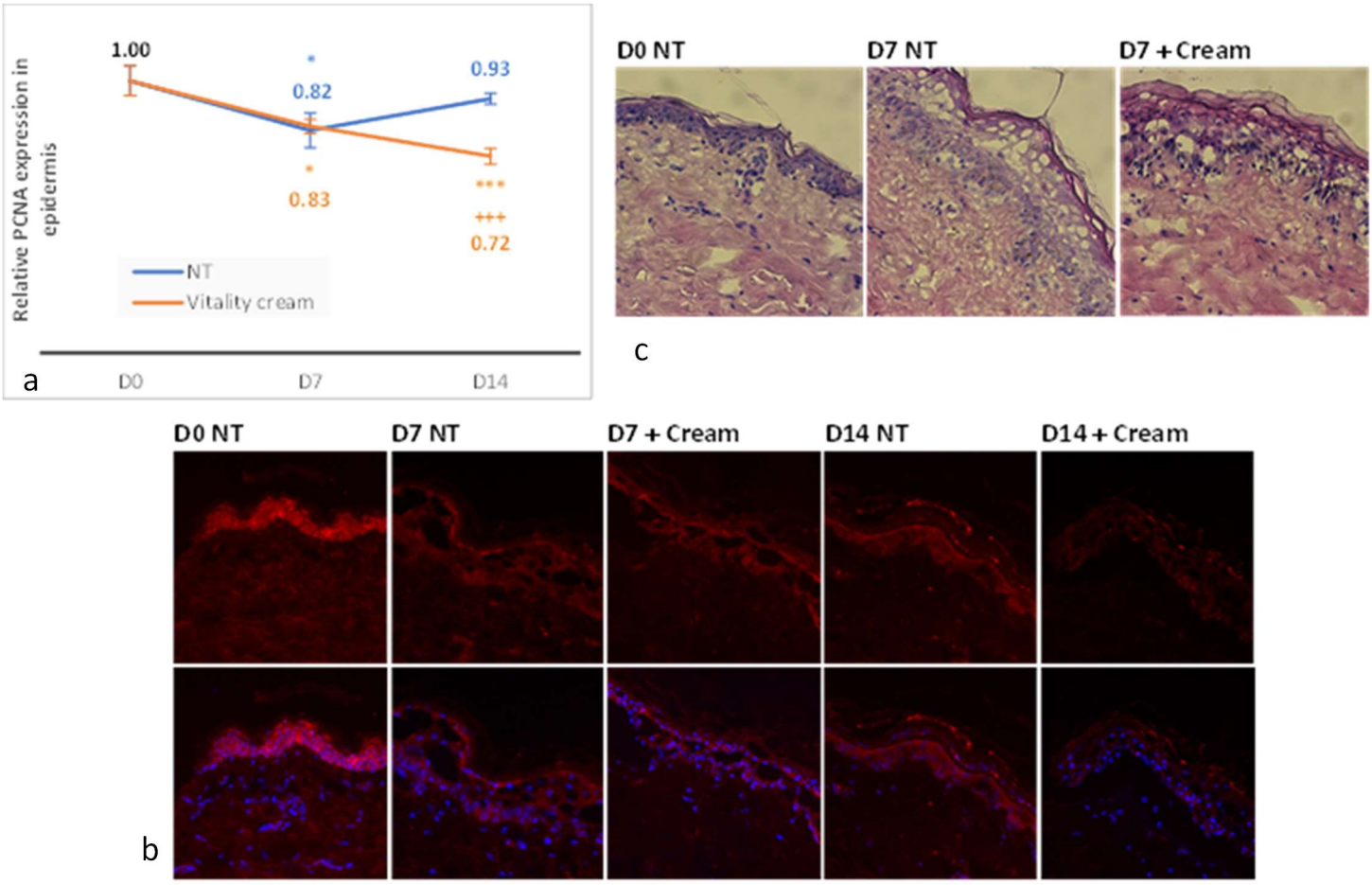


Figure 4 PCNA and hematoxylin labelling.

Wounded explants were treated with Biovive or untreated (NT). The explants where sectioned and stained after 7 or 14 days and compared to the control at J0. The mean fluorescence intensity was measured inside the epidermis (b). Expression levels were computed relatively to the control (a). The error bars represent the SEM. Statistical analysis was performed using T-tests against the untreated condition: (* p-value<0.05, ** p-value<0.005, *** p-value<0.001) against J0 and (+ p-value) against the same day untreated condition.

Hematoxylin/Eosin staining was performed on the control and after 7 days (c).

Examples of pictures of the stained sections for each condition are shown in (c). In the first row only the labelling of PCNA is shown in red. In the second row nuclei are also labelled in blue.

Moreover, to determine tissue integrity over the time of our ASEM, hematoxylin and eosin staining of the tissue cross-section was performed (figure 4). In the epidermis, we observed all the strata: basal, spinosum, granulosum and corneum. At day 7, while we observed some vacuoles in the granulosum strata of the ASEM, the basal, spinosum and corneum strata appearance seems to be similar to the day 0. Thus, the ASEM model showed a good viability at day 7 with a good connection on the dermo-epidermal junction. Cream treatment seemed to reduce the vacuoles in the granulosum strata and displayed a thicker stratum corneum. More, cream treatment seemed to increase the number of nuclei in the epidermis and the dermis after 7 days.

DISCUSSION

A new wound healing model covering the crucial switch with between inflammation and proliferation phase

Wound healing is a complex mechanism that perfectly coordinates 4 phases: hemostasis, inflammation, proliferation, and remodeling. Hemostasis phase begins immediately after the injury and promotes the release of pro-inflammatory cytokines as well as growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Then, the inflammation phase occurs followed by the proliferation phase that fills the wound by a re-epithelization process, repairing the vascular network and reconstructing the granulation tissue. Finally, the remodeling phase improves wound contraction and restores the normal structure and function of the epidermis and the dermis.

Preclinical/animal models of wound such as burn, excisional, incisional, and impaired wounds by key protein expression alteration seems to be the most relevant methods (*19*) However, on the one hand the animal models are not allowed in cosmetic domain and on the other hand European union wants to drastically reduce animal testing, the development of new in silico, *in vitro* or *ex vivo* models is flying high. In such context, monolayer and organotypic cultures are widely used to evaluate the wound healing effect of novel active ingredients in cosmetic claims. Unfortunately, the latter lack complexity and do not correlate with *in vivo* observation. Human *ex vivo* skin biopsies are the most complete model available. The presence of a complete functional skin barrier, a well-organized extracellular matrix, skin appendages, the 3 major skin cell types (melanocytes, keratinocytes, and fibroblasts) and immune cells such as Langherans cells allow us to investigate many skin pathways and is a reliable alternative for clinical test and animal models (*20*). Several explant-based wound healing models have been previously used to increase our knowledge on wound healing pathways and identify new therapies(*21*–*25*). These models allow the development of dermal substitute or anti-inflammatory treatment using IL10(*21*, *23*). These models are variable in the thickness of the wound and the culture media and the plate support (i.e., collagen embedded, well chamber inserts). However, none has specifically focused on the switch between proinflammatory phase and skin reparative phase (proliferation and remodeling). Thus, excessive and prolonged inflammation promotes wound healing delay and excessive scaring. Thus, the switch between proinflammatory and proliferative phases is crucial. A skin wound healing model that mimics perfectly this delicate transition is a prerequisite to screen effective products that accelerate this process. In this study we developed, optimized, and characterized a double excisional skin wound in dermis and epidermis model of that may mimics this switch.

The inflammation phase protects the wound from infections after the skin barrier disruption by the release of cytokines such as interleukin-1 (IL-1), platelet-derived growth factor (PDGF), to attract immune cells. These immune cells produce TNFα and IL6. The latter is a pivotal regulator of the inflammatory/proliferation switch by controlling MMPs protease that locally destroy extracellular matrix around the wound to promote the fibroblasts and keratinocytes proliferation and migration into the wound. MMP expression and activity are tightly controlled during wound healing. MMP-3 control matrix degradation but also regulates chemokine

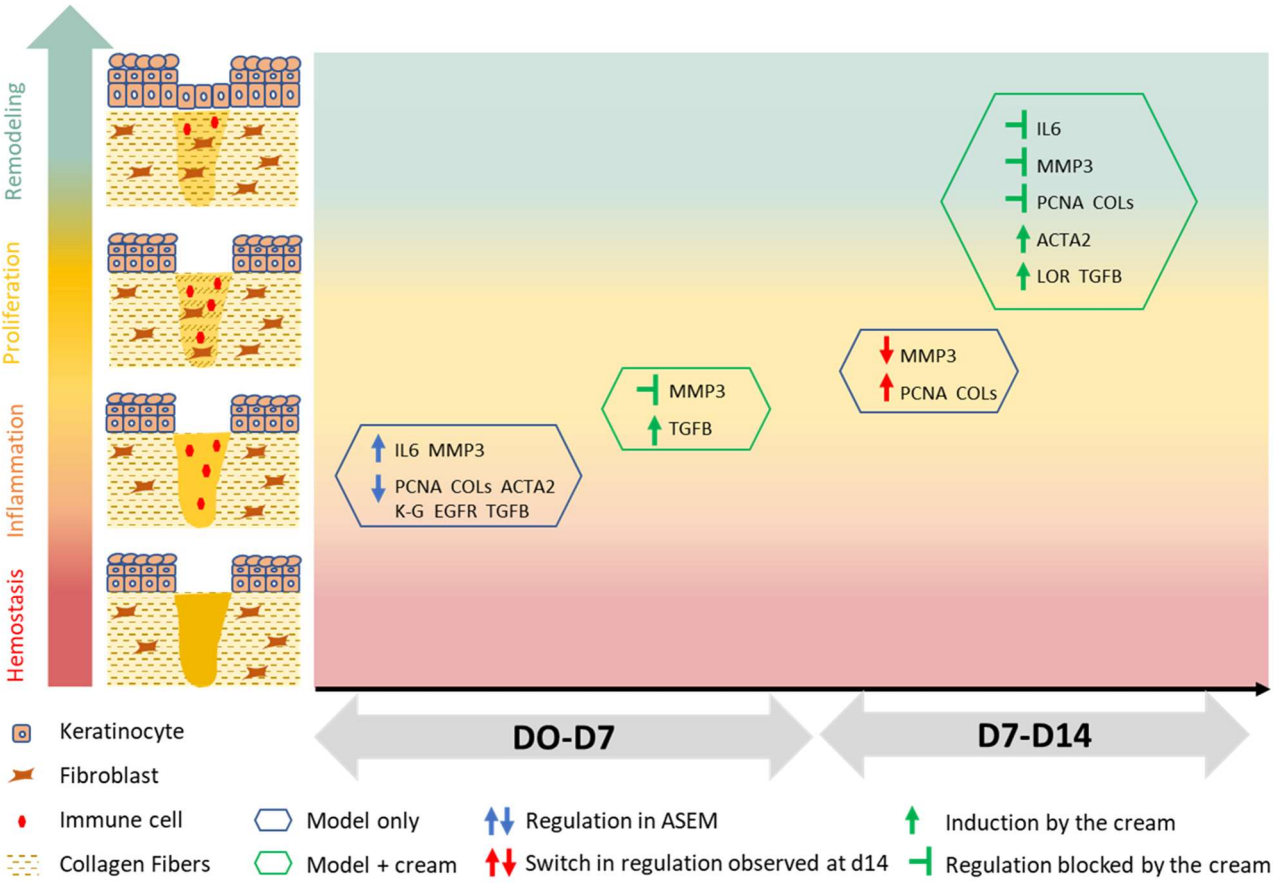


Figure 5 Overview of the model characterization and the cream effect on wound healing

*Schematic description of the wound healing process. The four major phases are presented on the left: Hemostasis, Inflammation, Proliferation and Remodeling. The figure shows the progress of the wound healing process in the model, with or without cream application, after 7 and 14 days. This progress is deduced from the relative expression of the genes of interest (and of the protein for **MMP3** and **PCNA**), according to their known role in the wound healing process: EGFR: early phase marker, IL6: inflammation marker, MMP3: matrix deposition marker, PCNA: proliferation marker, COL1A1, COL3A1 (COLs in the figure) and ACTA2: matrix and fibroblasts migration markers, LOR and FLG (K-D in the figure): Keratinocyte Differentiation markers we observed in C57BL/6J WT mouse wound healing at different time points*

expression and activity by degrading chemokines to remove them or produce receptor antagonists(*20*).

The figure 5 provides a schematic illustration of the mechanism of ASEM wound overtime. Taken together our data showed that from Day 0 to Day 7, our ASEM is clearly in the inflammation phase with the over-expression of IL6 and MMP3 combined to the repression of the specific markers of the other phases such as keratinocytes differentiation markers. From day 7 to day 14, MMP3 induction is blocked and the PCNA, a key proliferative inducer and the collagen begin to increase in expression.

To conclude, in our ASEM model, the Inflammation/proliferation switch seems to occur about 7 days after the wound. This observation is completely in accordance with what happens *in vivo*. Indeed, previous authors observed that in a mouse model (C57BL/6J WT) the model enter in proliferative phase at day 7 after the wound (*26*).

The BIOVIVE vitality cream accelerates the wound healing by reducing inflammation phase duration

The vitality cream was applied on ASEM and the same molecular markers of the different phases of wound healing were followed at RNA and proteins levels and compared to the control without treatment. The EGFR gene expression is repressed at 7 and 14 days, similarly to the control, probably because of an earlier expression during the wound healing process initiation (*27*, *28*)). IL-6 gene increased at 7 days and its levels remained stable until 14 days, but its expression is significantly lower compared to the control at these two times. In the same way, MMP3 gene expression and protein level are significantly decreased. MMP19 gene is also less expressed at 7 days compared to the control. These inflammatory phase molecular markers globally exhibit a lower expression level in the treated skin explant, which indicate that the inflammatory phase is likely finished before 7 days.

PCNA and COLIII genes are also repressed at 7 and 14 days but hematoxylin eosin-stained samples exhibit a greater density of nucleus in the cream-treated sample than in the control sample, suggesting that the proliferative phase would already have started at 7 days in the cream treated sample. COLIII gene expression, observed in the control sample, may have increased and then decreased between 0 and 7 days. PCNA expression is also precisely timed (*9*). Its expression takes place before the proliferation of cells in the epidermis, already observed at 7 days for the treated sample. This information indicates that the proliferative phase seems to have started before 7 days.

TGFβ2, α-SMA and loricrin gene expression increased together at 7 days and again at 14 days, compared to the control. TGFβ2 controls the proliferation of fibroblasts in the dermis, the extracellular matrix production and neo-angiogenesis. It was also showed to accelerate re-epithelization *in vivo* (*3*). α-SMA is a cellular marker of the differentiation of fibroblasts into myofibroblasts, which migrate into the wound and improve the wound contraction to close the wound (*29*). The expression of both genes in the cream-treated explant suggests that the proliferative phase occurs between 7 and 14 days, leading to the implementation of granulation tissue, but also the beginning of re-epithelization, as observed with the hematoxylin-eosin coloration. The wound contraction seems to start at 7 days. This was not observed with the control sample. Loricrin is a glycine-serine-rich insoluble protein, which is critical for the formation of the cornified envelopes and the development of the epidermis barrier. Its expression increase reflects the presence of well differentiated keratinocytes in the neo-formed epidermis (*30*) This data indicates that the re-epithelization begins before 7 days, under the stimulation of TGFβ2, and that the formation of the differentiated neo-epidermis starts before 14 days. The whole data suggest that between 7 and 14 days the wound starts to close with the implementation of the granulation tissue in the dermis and the beginning of the re-epithelization in the neo-epidermis. However, COLI, LAMA5 and FLG, protein markers of the remodeling phase, are still repressed at 14 days, indicating that the remodeling phase has just begun at 14 days.

Protein levels of PCNA (epidermis) and MMP3 (epidermis and dermis) and the gene expression of PCNA, EGFR, α-SMA, MMP3, MMP19, COLI, LOR, FLG and LAMA5 at 7 days in the treated sample are similar to those of control sample at 14 days. This suggests that the cream seems to accelerate the wound healing process of at least 7 days. Using this new model, the vitality cream shows to decrease the inflammation phase duration, stimulate the wound contraction and the

implementation of the granulation tissue and the neo-epidermis, compared to the control sample, and to stimulate the expression of TGFβ2 earlier, controlling the expression of differentiation markers like α-SMA (*31*), and proteins of the *stratum corneum* like loricrin. TGFβ2 gene expression were showed to increase after wounding, particularly in migrating epidermis, during the re-epithelization (*12*). However, its expression during wound healing naturally decreases with the age in old animal models, where wound healing process is more difficult and takes more time (*32*). The cream seems to stimulate the production of this hormone in the skin explant from a Caucasian woman of 55 years old and to accelerate the wound healing process. This suggests that this vitality cream with natural extracts may improve wound healing in elderly skins, where skin functions and wound healing process are usually impaired.

A natural ingredient synergy to improve wound healing

Natural extracts and oils that compose the active compounds of the cream may be synergistically involved in this improvement thanks to their respective bioactivities on human skin cells. This vitality cream is made with active extracts from natural origins, i.e., a bud extract (*Ficus carica, Prunus cesarus, Prunus persica*), *Camelina sativa* and *Helianthus annuus* oils and with young shoots extracts from *Hordeum vulgare* and *Oenothera biennis*).

Natural plant oils are commonly used in traditional medicines as emollient to reinforce the skin barrier and to form a semi-occlusive film on the skin, preventing dehydration and penetration of allergens and microorganisms (*18*). *Helanthus annus* oil, present in the cream, mainly consist of linoleic and oleic acid. It displayed beneficial effects in the treatment of atopic dermatitis skin lesion, reducing the severity of the disease. Regarding wound healing, it was showed to accelerate the healing process on a skin explant, increasing the wound contraction and diminishing the closure time of the wound compared to the control (*33*). These effects were also observed in this study at a molecular level. Other plant oils displayed the same characteristics on wound healing, notably coconut oil, which accelerates the re-epithelization, the neo-vascularization, and the deposition of the granulation tissue (*34*).

Oenothera biennis aqueous extracts were reported to suppress inflammation cytokines (IL1β, IL6 and TNFα) and to protect keratinocytes from oxidative stress by stimulating the production of antioxidant enzymes. It also promoted collagen matrix contraction in the wound and TGFβ type II receptors up regulation, increasing the production of extracellular matrix proteins (*35*). Moreover, β-glucan extracted from *Hordeum vulgare* displayed a differentiation activity on human dermal fibroblasts, stopping their proliferation and promoting the migration phenotypes of the cells. This feature complies with our results with the expression of TGFβ 2 and α-SMA, promoting the wound contraction and closure (*36*).

The bud extract, incorporated into the regenerating cream, was obtained from an aqueous extraction of *Ficus carica, Prunus cesarus, Prunus persica* buds*.* It is rich in polyphenol and complex polysaccharides (data not shown). Complex sugars and polyphenols were showed to improve wound healing. Indeed, glycosaminoglycans, heparin sulfates, dermatan sulfates and hyaluronans exhibit booster activities on wound healing, as there are used as molecular scaffold for tissue reconstruction. They also improve the acute and chronic wound closure, stimulate the proliferation of fibroblasts, their differentiation into myofibroblasts and regulate the neovascularization in synergy with TGFβ (*15*). Polyphenols such as quercetin showed to accelerate wound healing in rats by increasing the expression of IL-10, VEGF, TGF-β1, CD31, α-

SMA, PCNA, and GAP-43, and decreased the expressions of TNFα, as well as curcumin, which increases the expression of α-SMA in mice and decreased the expression MMP 9 in TNFα-stimulated fibroblasts (*17*, *37*). Similar effects were noted with the regenerating cream. Bud extracts were already described for their anti-inflammatory activities on skin disorders (*38*), but also for their ability to restore the natural gene expression in senescent cells. Indeed, *Sophora japonica* bud extract showed to suppress scaling, erythema and skin thickening in dermatose murine models, as well as hyperplasia and immune cell infiltration. An extract from *Populus nigra* buds allowed aged fibroblasts not only to recover expression of a catalase gene, observed in young cells, but also increase it more than 2-fold. It also modulated the gene expression of KLF, ZFP36L1 and E2F4 genes, which are involved in cell cycle regulation and in the renewal of epidermis, usually downregulated in senescent keratinocytes (*39*).

All the compounds present in the balm display a beneficial activity on skin protection and regeneration. They act in synergy, gathered in this cream, to accelerate the wound healing process by at least 7 days, by decreasing the inflammation duration, promoting the wound contraction and granulation tissue formation as well as improving the re-epithelization. Its ability to stimulate hormones and skin functions in aged skin allows to consider it for senior skin as booster of skin functions.

To conclude, ASEM model is of interest to highlight efficient cosmetic products able to accelerate inflammation and proliferative phase switch and avoid unsightly scars or chronic wound that are difficult to cure. Due to their multiple pharmaceutical properties, traditional medical herbs and natural extracts display different interesting properties, which can participate in wound healing *via* different mechanisms. The complex regenerative cream (Biovive, Laboratoires Gilbert) seems to accelerate the inflammation/proliferation switch and thus plays a key role in personal wound care.

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