Nourishing Wounds with Essential Amino Acids Accelerates Healing in Middle-Aged Rats

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Abstract

The principal therapy for cutaneous wounds is presently based on protecting the dermis from environmental assault, while simultaneously maintaining a humid environment. In order to improve wound healing, we explored the possibility of using a protective gel that also provides nutrients for wound repair cells. Providing essential amino acids from outside the wound is an unusual and yet potentially efficient support for the healing process. In skin wound repair of middle-aged rats, we found that nourishing wounds with essential amino acids shortened repair time, by reducing inflammation and influencing a full set of regulators of collagen-1 deposition and removal, such as transforming growth factor beta, its receptors and matrix metalloproteinases. Nourishing wounds with essential amino acids optimizes the quality and organization of collagen-1 deposition, thus accelerating the wound healing process compared with placebo. Topical application of essential amino acids appears to be a promising strategy in reducing healing time and potentially improving the quality of repairs.

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Introduction

The mechanism of skin wounds involve interaction between the environment and the dermis that in turn condition the efficiency of final healing. Depending on severity and the extension of wounds, the loss of liquids and proteins needs to be balanced, as this can become a possible threat to the integrity of the whole organism. The metabolic cost of wound healing is enormous due to the high amount of energy required and/or protein substrates consumed for re-synthesis of new skin and continuous remodeling requirements of stressed structures.[1]

The healing process occurs in three overlapping phases: inflammation, proliferation and remodelling. Each phase depends on various factors such as tissue type, age, infection, state of health and nutritional status.[2-10] Many molecules play important roles during the various stages of wound healing in modulating inflammation and tissue regeneration.[11-13]

Wound repair is characterized by a tight balance between collagen synthesis and catabolism. On the one hand, reduced synthesis of collagen slows repair, while hyper-production would on the other cause fibrosis. Therefore, any impairment of the normal reparative process can lead to either delayed healing or hypertrophic scars. As a result, any therapy needs to balance the modulation of molecules that regulate both collagen synthesis and/or its degradation.

Transforming Growth Factor beta-1 (TGF-β1) is a multi-functional peptide member of the TGF-β super-family of cytokines that plays an important role during development, homeostasis, disease, proliferation and regeneration.[3,14] This growth factor is usually secreted by fibroblasts as a complex with a latent TGF-β-binding protein, which is then removed by proteolytic cleavage. Subsequently, the active form of TGF-β can bind to the serine-threonine kinase receptor complex (type I, TGFβ–R1, and type 2, TGFβ–R2), so exerting its biological function.[15]

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As well as TGF-β1 and other proteins, fibroblasts also produce matrix metalloproteinases (MMPs), a family of Zn-dependent endopeptidases implicated in many biological processes.[16] MMP9 (or gelatinase-B), is a type IV collagenases considered to be a key enzyme involved in wound healing and tissue remodeling.[17,18] Prolonged and sustained MMP9 up-regulation, during the early healing phase, is also correlated to efficient re-epithelization.[19,20] On the contrary, down-regulation of MMP9 reduces active TGF-β1 and as a result several TGF-β1 driven responses, such as fibroblast contraction.[21] We can therefore see that the level of MMP9 expression is a useful parameter to assess the normal progression of wound repair.

There is a wide variety of dressings available for wound care,[22-25] although the most commonly available care products do not take into account the greatly increased cellular demand for nutrients, needed to generate new tissue and close the wound. As a result, a question that we consider pivotal, is whether it would be useful to consider wounds as an active site for refueling cells implicated in repair management. This would improve the healing efficiency achieved by "localized" nutrition.

The importance of nitrogen intake in producing collagen for skin wounds repair has been demonstrated in both young and mature rats. This demonstrates that age-related wound repair problems are mainly influenced by the mix of nutrients available to cells.[26]

In a study on an excisional wound model of aged rats, we demonstrated that local availability of the four amino acids (AA) needed for collagen synthesis (glycine, proline, lysine, leucine) accelerate wound closure.[27] In other work, we showed that diet supplementation with balanced mixture of essential-AA (EAA) prevents tissue damage caused by senescence in kidney and muscle tissue,[28,29] improving mitochondriogenesis and increasing tissue life span.[30] In addition, it has been demonstrated that malnutrition is associated with impaired wound healing.[31] Accordingly, we recently showed that the quality of nutrition and content of EAA are strictly connected to skin health and integrity of its protein components and that the most relevant nutritional factor for skin health is the prevalence of EAA.[32-34] Therefore, the balanced nutrition of injured tissues may be of fundamental importance to improve cell turnover and promote wound repair, thereby minimizing scar formation.

To test this hypothesis we examined the effects of topical "*nutrition of wounds*" by comparing a medication composed of balanced EAA mixture to a placebo medication, in a full thickness excisional model of dermal wound in middle-aged rats. We studied the modulation of key elements of wound repair, such as TGFβ1, TGFβ-R1, TGFβ-R2, MMP9 and collagen-1 (Col1A1) production, using Western blot (WB) and/or immunohistochemical (IHC) methods.

Materials and methods

Animals

The experimental protocol was approved and conducted in accordance with the Italian Health Ministry and complied with '*The National Animal Protection Guidelines'*. The Ethics Committee for animal experiments of the University of Brescia and the Italian Health Ministry also approved the procedures. Sixteen male Sprague-Dawley rats aged 12 months, weighing 445±30g, (From Envigo, Italy) were placed in a quiet, temperature and humidity controlled room and were kept on a 12/12-h light/dark cycle. All animals were fed a standard diet (Mucedola srl, Milan, Italy) and water *ad libitum.*

The animals were randomly divided into 2 groups: group 1. (n=8) dressed with 7% EAA ointment (expressly prepared by Nutriresearch s.r.l, Milan, Italy), and group 2 (n=8) dressed with the same vehicle as the previous dressing (Placebo). The composition of the dressings is summarized in Table 1. The EAA-based formula contained Cystine, N-Acetylcisteine and Methionine to match sulphur AA needs without any metabolic toxicity. Tyrosine was also present, when calculating Phenyl-Alanine needs, as Tyrosine is only considered a non-EAA for the liver and partially for the kidneys, given that it is derived from enzymatic hydroxylation of Phenyl-Alanine. Consequently, it is considered fully essential in all other cells lacking this specific hydroxylating enzyme. L-Ornithine α-Ketoglutarate was also provided with EAA ointment, both to optimize nitrogen metabolism and Proline synthesis,[35] and as a mitochondrial prebiotic.[36,37]

All wounds were monitored, cleaned and medicated twice daily.

	%	EAA (g/100g)	Placebo (g/100g)
L-Leucine	13.53	0.947	-
L-Lysine	11.6	0.812	-
L-Isoleucine	9.65	0.675	-
L-Valine	9.65	0.675	-
L-Threonine	8.7	0.609	-
L-Cystine	8.2	0.574	-
L-Histidine	11.6	0.812	-
L-Phenyl-alanine	7.73	0.541	-
L-Methionine	4.35	0.304	-
L-Tyrosine	5.8	0.406	-
L-Tryptophan	3.38	0.236	-
N-Acetylcisteine	0.97	0.068	-
L-Ornithine α Ketoglutarate	2.42	0.169	-
Vehicle			
water	81.85	76.12	81.85
vegetal glycerin	17	15.81	17
glucomannane	1.15	1.07	1.15

Table 1. EAA Composition of Dress Ointment (7% in EAA) vs Placebo.

Wounds and dressing. The cutaneous excisional wound model is considered adequate for biochemical and histological assessment of healing[38] and was performed as previously described.[27] Briefly, each rat was anesthetized with an intramuscular injection of Zoletil (30mg/kg) (Virbac, Carros Cedex, France), their dorsal surface skin was shaved and cleaned with 0.1% iodine alcohol. Four full thickness round wounds with a diameter of 5 mm (area about 19.5mm^2), two on each side of the dorsal midline, were made with a surgical biopsy punch (Kay Medical ltd., Seki City, Japan). Wounds were not stitched and were left uncovered, the animals were allowed to recover and were housed individually.[39] 0.5 g of medication ointment was then weighed and applied to each wound. The pH of dressings was the same for both cohorts. A skilled veterinary doctor monitored the animals daily.

Rate of wound closure. Each wound was photographed at day 0 (day of wounding), and subsequently after 3 and 10 post-wounding days (*pwd*). Following Ring et al. [40] the perimeter of each wound was traced onto a glass slide with a pencil. The wound area (mm^2) was calculated from the perimeter traced using image analysis software (Image Pro Plus, Immagini e Computer, Milano, Italy). All wound areas were expressed as 100% on the day of wounding (day 0) and on subsequent days, were expressed as a percentage of the original area, using the following formula.

Rate of wound area = wound area (3 or 10 pwd)×100% / wound area day 0

Experiment termination. After 3 and 10 *pwd*, the animals were euthanized under deep anesthesia. The wounds were excised and bisected at the mid-point. The half-wound samples were frozen in liquid nitrogen and stored at –80°C for molecular studies. The other half was immersed in immunefix for 12h at 4 °C, then washed in PBS (0.2M, pH7.4) and processed according to standard procedures for paraffin embedding.

Histology. Five-µm-thick sections were cut by microtome and stained with haematoxylin and eosin (H&E). At 10 *pwd,* the neutrophils and fibroblasts density, expressed as the number of cells per 100 square micron of wound area (nr/100µ^2), were measured using a bright field optical microscope (Olympus BX50, Tokyo, Japan) from ten fields of each group.

Histochemistry. Collagen was evaluated with a picrosirius stain (Sirius-red) as previously described.[41] The tissue sections were analysed for collagen organization and fibrosis using an optical microscope under polarized light. Under a polarizer filter, collagen fibres of various thicknesses appeared as being stained differently. Although the birefringent colour is more a measure of collagen fibre size than of collagen type, usually the thicker and denser type I collagen fibres were detected as orange to red, whereas the thinner type III collagen fibres appeared yellow to green.[41-44]

Western blot analysis. Total proteins were extracted from tissues in lysis buffer (50 mM Tris-HCl, pH7.8, 1% Triton X100, 0.1%SDS, 250mM NaCl, 5mM EDTA, 100mM NaF, 2mM NaPPi, 2mM Na_3VO_4, 1mM PMSF). The crude lysate was centrifuged at 16000xg, the supernatant was recovered and assayed for protein concentration with the Bradford Assay (Bio-Rad Laboratories, Milano, Italy). Fifty

micrograms of protein extracts were run on a 7.5% SDS-PAGE for Col1A1 or 12% SDS-PAGE for TGFβ1, TGFβ-R1, TGFβ-R2, and transferred onto a PVDF membrane (Millipore, Milano, Italy). The membranes were stained with Ponceau Red (Sigma-Aldrich, Milano, Italy), in order to check for transfer and were then blocked at room temperature for 2 hours with 10% non-fat dry-milk in Tris-Buffered Saline (50 mM Tris-HCl pH 7.5, 150mM NaCl) plus 01% Tween20 (TBST). Subsequently, the blots were washed briefly and incubated over night at 4°C with rabbit polyclonal primary antibodies directed against TGFβ1 (sc-146), TGFβ-R1 (sc-398), TGFβ-R2 (sc-400), all provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Col1A1 (Thermo Scientific, Rockford, IL, USA), diluted 1:500 with 5% non-fat milk in TBST0.1% Tween20.

The membranes were then washed three times for 10 minutes with TBST. They were then incubated for one hour at room temperature, with anti-rabbit HRP-conjugated secondary antibody (Bio-Rad Laboratories, Milano, Italy) diluted 1/2000 in TBST containing 5% non-fat milk. The membranes were washed three times for 10 minutes, incubated in Super Signal West Pico (Thermo Scientific, Rockford, IL, USA) chemo-luminescent substrate and exposed to a Chemi-Doc XRS plus System (Bio-Rad Laboratories, Milano, Italy) that is capable of a high linear dynamic range and of showing saturation of the most concentrated band, thus enabling linear quantification.). The expression levels of the target proteins were normalized versus actin (rabbit polyclonal, sc-1616R), except Col1A1 that was normalized versus-tubulin (mouse monoclonal, clone 4G1, sc-58666). Both control antibodies were provided by Santa Cruz Biotechnology. Each protein sample was loaded in gel in triplicate and the experiments were repeated three times.

Immunohistochemistry (IHC). The sections were incubated overnight with primary anti-TGFβ1 (sc-146), anti-TGFβ-R1 (sc-398), anti-TGFβ-R2 (sc-400), anti-Col1A1 (sc-25974), anti-MMP9 (sc-6840), polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) diluted 1:100 in PBS. They were then visualized with a rabbit ABC-peroxidase staining system kit (Santa Cruz Biotechnology), and mounted with DPX. The reaction product was visualized using 0.3% H_2O_2 and DAB at room temperature. The primary antibody was omitted for the control cohort.

Statistical analysis. Staining intensity of IHC-treated samples was evaluated quantitatively using an image analysis program (Image-ProPlus 4.5.1). The integrated optical density (IOD) was calculated at the marginal and the central wound area by measuring five fields for each sample. Statistical analysis using one-way ANOVA followed by the Student Newman-Keuls (SNK) test or Student's t-test was performed. The intensity of WB bands was evaluated quantitatively using a computer-assisted densitometer (ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA), normalized versus the actin or tubulin internal control. $P<0.05$ was considered significant.

Results

All animals survived the surgical procedures and there were no complications and/or wound infections. Furthermore, we did not observe any changes in animal weight, food and water consumption during treatment (data not shown).

Macroscopic appearance of wounds. Ten *pwd*, there were marked differences in the macroscopic appearance of the healed wounds. At this time, it was quite difficult to locate the sites of the EAA treated wounds. In contrast, the sites of the wounds treated with Placebo were clearly visible because the open wound areas were still evident and surrounded by a white fibrotic ring.

Wound closure time. To evaluate whether treatment with different dressings influenced wound closure time, wound measurements were taken at the end of treatment for all animals, after gently removing the crust whenever present. The healing time of wounds dressed with EAA, was markedly shorter when compared to Placebo-treated wounds (Figure1A and 1B).

Histological findings. Epidermis. H&E staining was performed on the wounds harvested at 10 *pwd* (Figure 2A-C). Ten *pwd*, re-epithelialization was complete in the EAA-treated wounds, whereas the Placebo-treated wounds were still undergoing the re-epithelization process. However, the organization of epithelial layers was very different between groups. EAA-treated wounds had a well-formed epithelium and increased interdigitation between the epithelial and the dermal layers without clefts. No

damaged cells or atypical keratinocytes were found. The basal layer was regular and well organized (Figure 2B). Placebo-treated wounds were the deepest and showed vacuolar degeneration of basal cells, dyskeratotic bodies and the presence of apoptotic-like keratinocytes in the basal and spinosum layers (Figure 2C)., We also observed clefts and spaces at the epidermal-dermal junction in various wounds of the Placebo-treated group.

Dermis. Ten *pwd*, the wounds were also stained with Sirius-red to highlight the orientation of collagen fibers in the dermis (Figure 2D-F). Wounds dressed with EAA showed abundant and thick collagen fibers (orange-red color at polarized light) in the inner and deeper layers of the newly formed tissue. These fibers were oriented in an orderly manner, closely arranged and parallel to each other (Figure 2E). On the contrary, the Placebo-treated wounds had fewer collagen fibers, with a marked difference in organization and orientation. Indeed, they was thinner, shorter, spread out and without any organized orientation when compared with EAA-treated ones (Figure 2F).

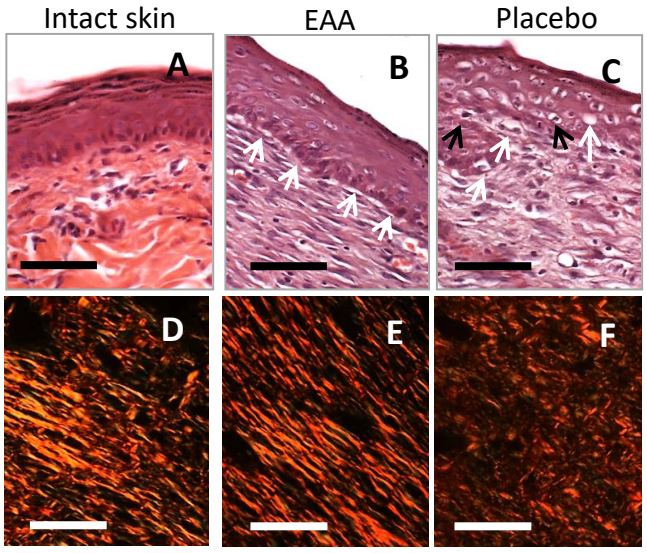

Figure 2. Representative pictures of E/H (A-C) and Sirius-red (D-F) staining (polarized light) for collagen fibers of wound area according to dressing at 10 *pwd* compared to uninjured (intact) skin. Similar to intact skin (A), EAA dressing (B) favored the generation of thick and organized epidermis layers. No damaged cells or atypical keratinocytes were found. The basal layer is regular and well organized (white arrows). In addition, EAA treatment favored the production of regularly oriented and compact network of collagen fibers (E) like those in intact skin (D). On the contrary, Placebo-treated wounds showed marked disorganization of basal layer, vacuolar degeneration of basal cells (white arrows), with the presence of apoptotic-like keratinocytes (black arrows) in basal and spinosum layers (C). Placebo-dressed wounds reduced collagen fibers that appears thinner, shorter, without any organized orientation and often packed (F). A-C scale bar =100µm. D-F scale bar =30µm.

Fibroblasts and neutrophils density. Ten *pwd*, the fibroblast density (nr/100µ2) in EAA-treated wound was significantly higher than the Placebo-treated wounds (Figure 3A). On the contrary, neutrophil density (nr/100µ2) was lower in EAA-treated wounds than Placebo-treated ones (Figure 3A and B).

Western blot (WB) and immunohistochemical (IHC) findings

TGF-β1. WB data showed that three *pwd*, the Placebo-treated wounds had higher levels of TGF-β1 than the EAA-treated ones. Ten *pwd*, we observed a trend towards a decrease of TGF-β1 in both groups (Figure 4A and 4B). The anti-TGF-β1 IHC staining confirmed the WB data (Figure 4C).

TGFβ-Receptors. Because the activity of TGF-β1 strictly depends on binding to its receptors, in order to promote collagen production and cells function, we also assayed the expression level of the TGF-β receptors (TGFβ-R). WB data showed that three *pwd*, the expression of TGFβ-R1 did not vary between the two groups. Ten *pwd*, we observed a strong downward trend of TGFβ-R1 expression in Placebo-treated wounds, whereas the level did not vary in the EAA-treated ones (Figure 5A and 5B).

Three *pwd*, TGFβ-R2 expression was considerably higher in the EAA treatment compared to the Placebo. At ten *pwd*, we observed significant decreases of –R2 expression in the EAA treatment, whereas there was no change in Placebo-treated wounds (Figure 5A and 5C).

At three *pwd*, the TGFβ-R1/TGFβ-R2 ratio in EAA treated wounds was 1:1 compared to approximately 2:1 in the Placebo treatment

due to -R1 over-expression. Ten *pwd*, the ratio increased (>1) as a result of excess -R1 levels in EAA-treated samples. With the Placebo treatment this ratio was <1 (Figure 5D).

Col1A1. As TGFβ1 plays a pivotal role in promoting collagen synthesis, we also assayed the modulation of Col1A1. WB data showed that at three *pwd*, EAA- and Placebo-treated wounds had scarce expressions of Col1A1. Ten *pwd*, the Col1A1 levels were very strongly up-regulated in the Placebo but less so in the EAA treatment (Figure 6A and 6B). The IHC-staining confirmed the WB data (Figure 6C).

MMP9. The balanced management of ECM during healing phases is regulated by MMP activity. In particular, MMP9 is a collagenase that when in excess, can destroy the ECM environment, disrupt resident cells and stimulate inflammation, thus playing an important role in tissue remodeling.[45,46] We therefore decided to assay its expression by IHC. The variation of anti-MMP-9 staining is summarized in figure 7. At three *pwd*, the staining did not vary significantly between the two groups. However, at ten *pwd*, the wounds medicated with EAA showed a significant reduction of staining compared to three *pwd* and to Placebo. This reduction was observed in wounds treated with Placebo only to a lesser extent.

Discussion

We have shown that a wound nutritional approach providing EAA with the dressing improved and accelerated their repair, modulating collagen I fibre production and organization, as well as TGF-β1, TGFβ-R1, TGFβ-R2, and MMP-9 expression. Wound repair is a complex biological process as multiple biochemical pathways become immediately and simultaneously activated. In our opinion, wounded tissue needs an adequate and constant nutritional supply supporting a favourable metabolic environment. The rationale for treating wounds with hydro-colloidal medication enriched with a particular stoichiometric composition of the AAs most contained in collagen, was reported in 2008.[47] Accordingly, it has also been demonstrated that topical therapy of wounds in aged rats, using a gel containing the four AA mostly required for collagen production, shortens healing time by modulating the expression of the major controlling elements of repair (TGFβ, eNOS and iNOS, VEGF) and effectively reduces inflammation.[27] The healing process also needs the participation of different types of cells, contributing to different ECM components.[48] However, an uncontrolled healing process could lead to scarring or fibrosis, due to excessive synthesis and accumulation of ECM proteins and loss of tissue homeostasis.

Collagen, the most abundant protein in the human body (30% of all body proteins), also represents around 50% of skin weight and its synthesis requires a lot of energy.[49,50] Collagen is a natural substrate for cellular attachment, growth and differentiation, so playing a crucial role in the healing of skin and other tissue wounds, and so the substitution of connective tissue matrix is necessary. As a result, both artificial and natural polymers have been used to promote dermis reconstitution.[51] Our data show that this EAA-based medication induced the early production of well-oriented collagen fibers, thus creating a more favorable environment for the rapid reconstruction of the skin by reducing closure time.

The fibroblast invasion of wounds and deposition of a collagen-based ECM has been termed fibroplasia. However, neo-vascularization also occurs and this overall process is called granulation tissue formation. Generally, this phase begins after 3-5 *pwd* and persists for 10-12 *pwd*, during which time there is a rapid synthesis of type I (80-90%) and type III (10-20%) collagen and an associated increase in the tensile strength of the wound.[52] Type III collagen is usually considered to be localized in the thin reticular fibrils while type I collagen is found in the larger fibrils.[53] The availability of all EAA promotes the production of thicker type I collagen fibers. Placebo medication supports the intense production of Col1A1 at 10 *pwd*, but impairs adequate building and organization of collagen fibers. In our opinion, this is a point that needs to be investigated further. Indeed, it is possible that the strong increase in Col1A1 production could lead to a slow, gradual but uncontrolled increase of mature collagen fiber production at a later point over 10 *pwd*. The impairment of collagen fibers production and/or its incorrect orientation could be a major difference between a highly organized dermis and a fibrotic scar. As such, we speculate that inadequately nourishing wounds could increase the risk of fibrotic scarring.

Collagen production by fibroblasts and the release of other ECM components, such as

proteoglycans and fibronectin, depends on TGF-β1 signaling.[54] Indeed TGF-β1 is believed to play an important role in wound repair, as it is a key regulator of the production and remodelling of the ECM through its effect on mesenchymal cells. Furthermore, it also stimulates angiogenesis, fibroblast proliferation and myofibroblast differentiation.[55] Thus, the variation of TGF-β1 synthesis during the healing phases plays a pivotal role in controlling wound closure.

A study on TGF-β1 deficient mice, demonstrated that the absence of this mediator can be compensated for during early stages of wound repair, but not at later stages.[56] Furthermore, mice treated with a neutralizing anti-TGF-β1 antibody exhibit severely impaired late stage wound repair.[57] Thus, the continuous supply of an adequate amount of TGF-β1 throughout the wound repair process is crucial for appropriate wound healing and aberrant TGF-β1 levels of expression is associated with wound healing defects.[3]

During tissue repair, the wound is contracted because local fibroblasts undergo a phenotype change from their normal relatively quiescent state, to the proliferative and contractile phenotype characteristics of smooth muscle cells, termed myofibroblasts. These cells can be induced by TGF-β1 to express α-SM actin, gaining the ability to contract, which plays an important role in dermis repair after injury.[58-60] The quick wound contraction observed in EAA dressed wounds also suggests the probable early modulation of fibroblast/myofibroblast transition. Further studies are in progress to verify this hypothesis.

Our data show that during later phases of healing, the EAA dressing reduced TGF-β1 expression compared to Placebo dressed wounds. This would suggest both an acceleration and improvement in efficient wound healing regulation. Indeed, although TGF-β1 is fundamental for wound healing, when excessive, it inhibits closure, acting as a negative regulator of re-epithelialization.[7] The reduction of TGF-β1 in EAA-treated wounds, would suggest the importance of its behaviour patterns in controlling and modulating collagen synthesis and perhaps fibroblast/myofibroblast transition. As a result, the protracted up-regulation of TGF-β1 observed in Placebo-treated wounds, is expected to lead to a higher likelihood of wound fibrosis, given that TGF-β1 is known to be intrinsically involved with the formation of scar tissue. It has also been shown that early and protracted up-regulation of TGF-β1 and elevated collagen synthesis detected in hypertrophic scars and keloid fibroblasts, are related to fibrosis.[61-63] We can therefore presume that blunting TGF-β1 expression at the appropriate moment in adult cutaneous wounds could also favourably control scarring.

To perform its function, TGF-β1 must bind to specific membrane receptors. Type I Receptor (-R1; 65-70 kDa) and Type II Receptor (-R2; 85 kDa) are trans-membrane serine-threonine kinases and are simultaneously signal transducing receptors. Type III Receptor (-R3; betaglycan) is non-signaling and functions mainly to present TGF-β to -R2.[63] Indeed, TGF-β1 initially binds to -R2, which has a constitutively active kinase. Subsequently, -R1 binds to the TGF-β1 molecule and becomes phosphorylated by -R2. -R1 and -R2 form a complex (probably a heterotetramer) and so generating a signal.[64] Given that TGF-β1 must bind to both receptors to perform its function, we assessed the expression levels of both of these molecules.

Previously, TGFβ-R1 was shown to be the main mediator of the TGF-β effects on ECM induction, whereas TGFβ-R2, possibly in conjunction with TGFβ-R1, mediates the anti-proliferative effects of TGF-β.[65] However, TGFβ-R2 levels may be less critical for collagen production.[66] While TGFβ-R2 levels are more potently induced earlier during the initial proliferative stages of wound healing and then decline, TGFβ-R1 levels remain high at the end of the proliferative stage, and at the onset of the synthetic phase, during which matrix deposition increases.[67] In accordance with the literature, our data suggest that TGFβ-R synthesis may be differentially regulated by EAA treatment during wound healing. Indeed, EAA medication could alter the TGFβ-R1/TGFβ-R2 ratio and subsequently rebalance proliferative versus matrix-inducing effects of TGFβ1. Furthermore, an increased TGFβ-R1/TGFβ-R2 ratio may underlie aberrant TGFβ signaling in scleroderma skin and contribute to elevated basal collagen production.[66] We have shown that in EAA-treated wounds, ECM production is regulated appropriately, suggesting possible prevention of scar formation.

Fibroblasts, as well as TGF-β1 and numerous other factors, also produce metalloproteinase (MMPs), a family of Zn-

dependent and neutral endo-peptidases implicated in many biological processes.[16] Among these, MMP9 is considered a key enzyme involved in wound healing and tissue remodeling.[17,18] MMP9 is mainly produced by dermal fibroblasts and inflammatory cells.[68-70] MMPs are secreted to facilitate the clearance of foreign and noxious agents.[45,71] These have been detected at the migrating epithelial front[72,73] and have been shown to be able to activate latent TGF-β and increase its effects by disentangling it from latent TGF-β binding protein.[74,75]

We found intense MMP9 immunostaining in both groups during the early phase of wound repair. Indeed, prolonged and sustained MMP9 up-regulation in the early healing phase, implicates this enzyme in re-epithelization.[19,20] On the other hand, down-regulation of MMP9 reduces active TGF-β1 and reduces several TGF-β1 driven responses such as fibroblast contraction.[21] In the later phase of wound closure with EAA medication, we observed a significant decrease of MMP9 staining. In our opinion, this reduction is linked to the fact that wounds treated with EAA ointment close more rapidly and collagen fibers are thicker and more organized. As a result, large amounts of MMP9 for tissue rearrangement are no longer necessary.

Interestingly, tissue extracts of abnormal scars have very low levels of MMP9 compared to normal skin.[76] However, when in excess, MMP9 can also disarrange the ECM environment, disrupt resident cells and stimulate further inflammation, so playing an important role in the modulation of tissue remodeling.[45] Our data showed that MMP9 is probably not a fully limiting factor for wound healing since MMP9's role may be multifaceted. Indeed, experimental study with MMP9-deficient mice showed enhanced re-epithelialization, implying that MMP9 could play an inhibitory or regulatory role in epidermal wound-healing and so it is not indispensable for wound closure.[77]

Recently, in work on diabetic feet, it was found that fibroblasts with high levels of MMP9 decreased proliferation rates, activity, migration and secretion of collagens. This would suggest that MMP9 inhibits fibroblasts and in turn influences healing.[78] It has also been suggested that high MMP9 rates may accompany inflammation and poor wound-healing of diabetic foot ulcers.[79] This is probably because it is related to increased inflammation, given that MMP9 is expressed mainly by neutrophils and macrophages. On the contrary, some experimental data in athymic nude mice suggest that MMP9 activity is a necessary condition for optimal, scarless repair.[80] Our data contradict this hypothesis, since we observed a tendency for MMP9 expression to decrease towards the end of the experiment, although this was in healthy adult rats. It should however be pointed out that the longest observation time-point in our study was only 10 days, so we cannot rule out further fluctuations of MMP9 after longer treatment periods.

In our opinion, this nutritional approach to wound-care with EAA could provide a safe and effective strategy to accelerate and improve the quality of both acute and chronic wounds repair in humans too. This could be particularly efficient in patients with increased nutritional needs, improving their quality of life and reducing management costs.

Conclusions

EAA wound treatment, promotes optimal nutrition to the environment that could accelerate new tissue generation, thus shortening healing time.

Declaration of Interest

The authors declare no conflict of interest except Francesco S. Dioguardi who is inventor and owner of US patents n°:
-US6218420 B1. Compositions based on amino-acids, for preventing and treating alimentary overloads in conditions of elevate body nitrogen requirements, without causing calcium loss.
-US7973077 B2: Amino acid based compositions for the treatment of pathological conditions distinguished by insufficient mitochondrial function.

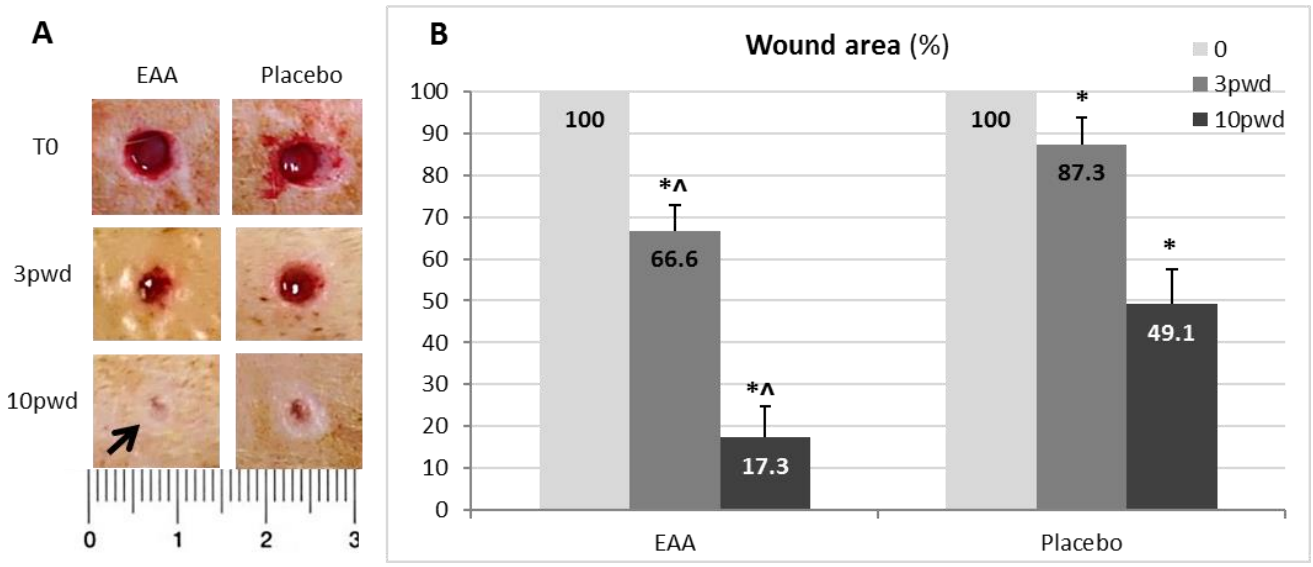

Figure 1. (A) Representative dorsal wounds at different times, according to treatment. At ten *pwd*, the wound closure is almost complete in EAA dressed animals (arrow). (B) Percentage (mean±sd) of wound area according to dressing. Original wound size (pale grey column, day 0) and after 3 *pwd* (middle grey column) and 10 *pwd* (dark grey column). * *P*<0.01 intra-group: ^ *P*<0.01 inter-groups.

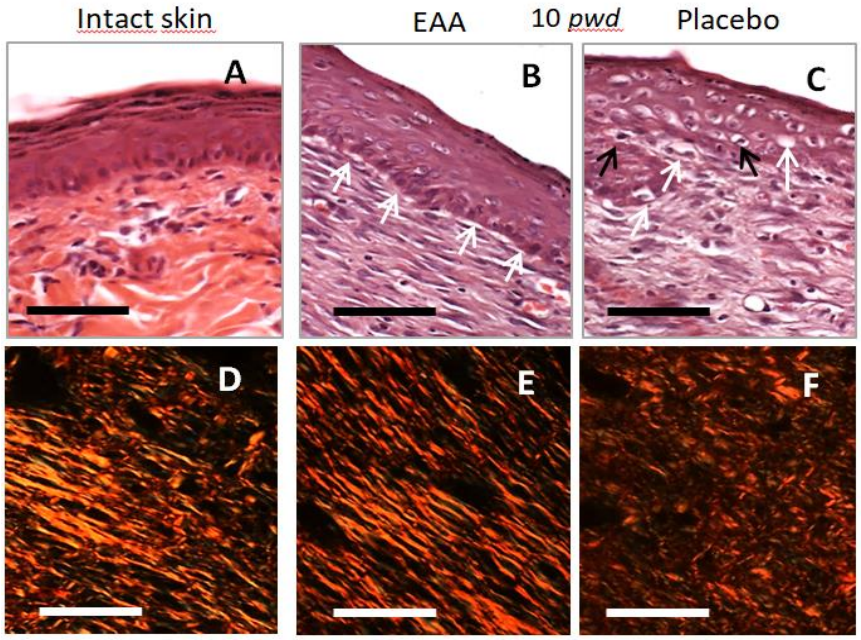

Figure 2. Representative pictures of E/H (A-C) and Sirius-red (D-F) staining (polarized light) for collagen fibers of wound area according to dressing at 10 pwd compared to uninjured (intact) skin. Similar to intact skin (A), EAA dressing (B) favored the generation of thick and organized epidermis layers. No damaged cells or atypical keratinocytes were found. The basal layer is regular and well organized (white arrows). In addition, EAA treatment favored the production of regularly oriented and compact network of collagen fibers (E) like those in intact skin (D). On the contrary, Placebo-treated wounds showed marked disorganization of basal layer, vacuolar degeneration of basal cells (white arrows), with the presence of apoptotic-like keratinocytes (black arrows) in basal and spinosum layers (C). Placebo-dressed wounds reduced collagen fibers that appears thinner, shorter, without any organized orientation and often packed (F). A-C scale bar =100µm. D-F scale bar =30µm.

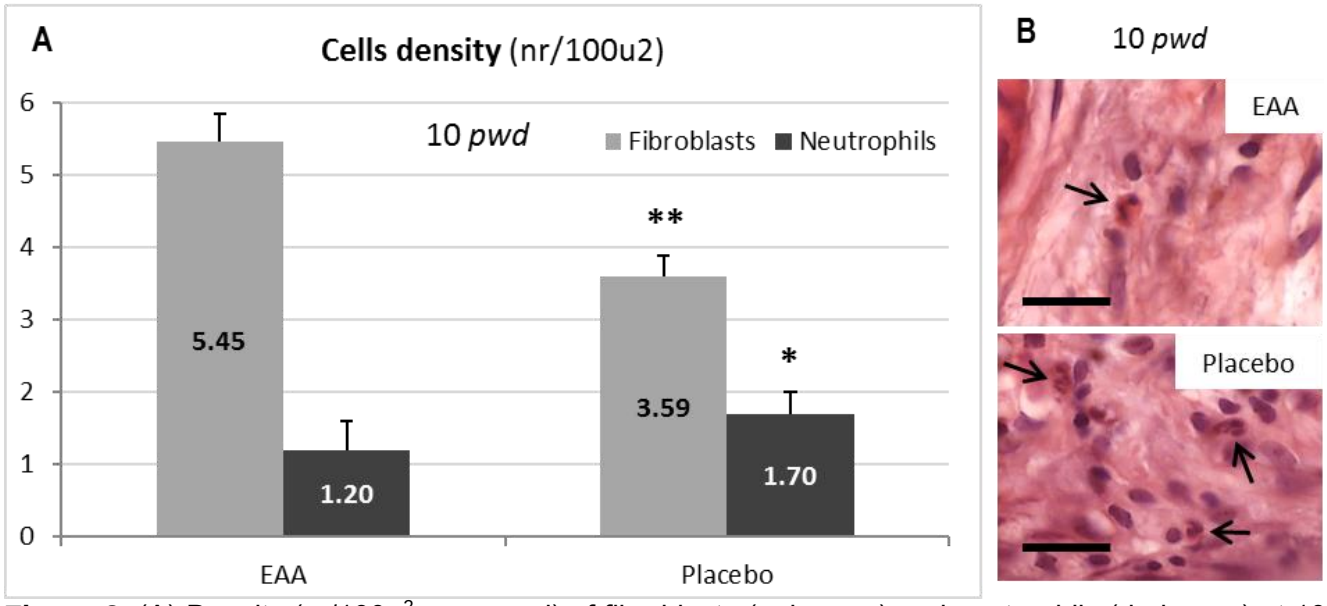

Figure 3. (A) Density (nr/100μ^2, mean±sd) of fibroblasts (pale grey) and neutrophils (dark grey) at 10 *pwd*. The EAA treatment promoted the increase of fibroblasts and decrease of neutrophils compared to Placebo-treated wounds. *P<0.05; ** P<0.01. (B) representative pictures of neutrophils (arrows) according to treatment (E&H staining) at 10 *pwd*. Scale bar = 50μm.

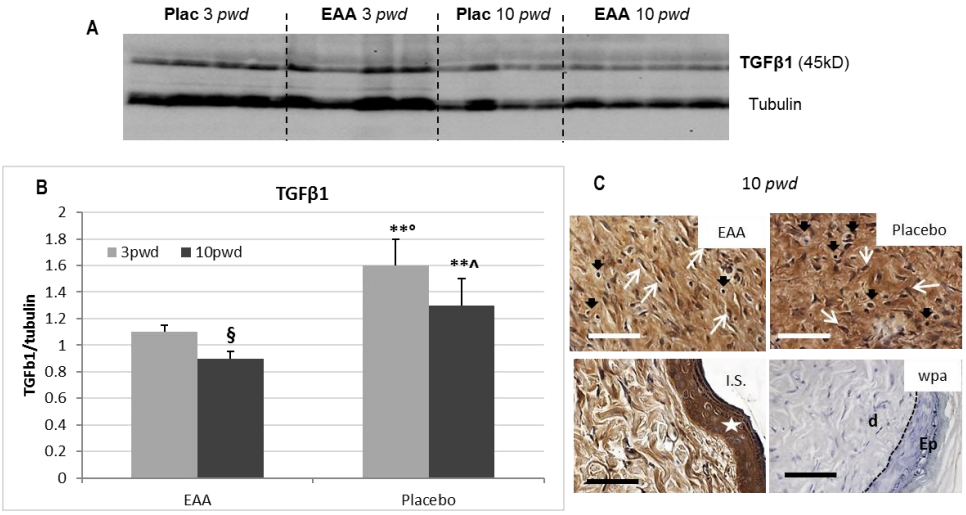

Figure 4. TGFβ1. (A) Representative bands of WB using anti-TGFβ1 antibody according to treatment and times. (B) WB data shows that EAA treatment early restrain TGFβ1 expression that decreased much more after 10 *pwd* compared to Placebo-treated wounds. § and ^ P<0.05 vs 3 *pwd*. ** P<0.01 vs EAA. Data are expressed as mean (±sd) from 3 experiments for each treatment and representative images of the blot bands are shown. (C) Representative pictures of IHC for anti-TGFβ1 at 10 *pwd*. EAA dressed wounds shows moderate but diffuse staining of fibroblasts. Sometimes more intense staining was seen. The nuclei of fibroblasts are elongated and regularly distributed (white arrows). Neutrophils and macrophages are present (black arrows). Placebo-dressed wounds showed more intense staining uniformly distributed. The nuclei of fibroblasts are large, irregular (white arrows) and frequently grouped. Neutrophils and large macrophages are present (black arrows). I.S. = intact skin, in dermis intense staining is mainly concentred in some limited cytoplasmic areas of fibroblasts. Conversely, all epidermis layers are uniformly and intensely stained (white star). wpa = without primary antibody. Ep = epidermis; d = dermis. Scale bar = 30μm.

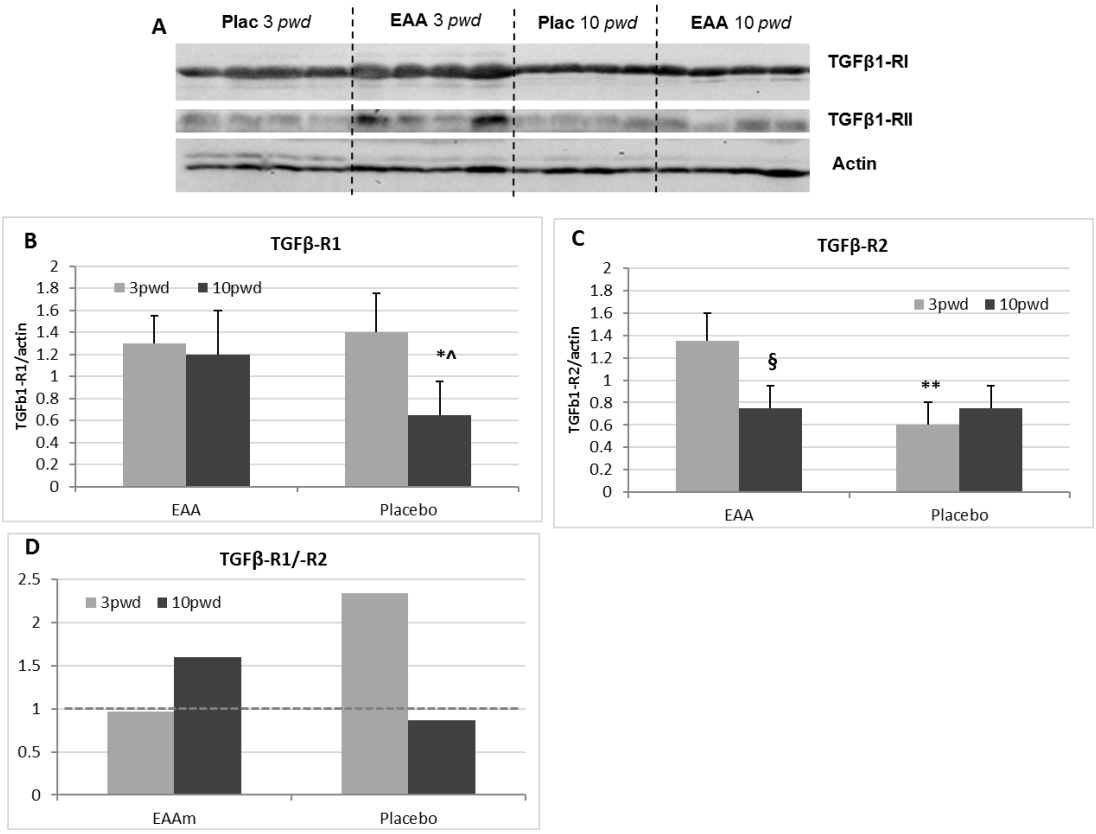

Figure 5. (A) Representative bands of WB using anti-TGFβ receptors antibody according to treatments and times. (B). TGFβ-R1 WB data showed that at 3 *pwd* its expression did not change between groups. Ten *pwd*, the –R1 expression decreased strongly in Placebo-treated wounds. ^ P<0.05 vs 3 *pwd*. * *P*<0.05 vs EAA. (C) TGFβ-R2 WB data shows that 3 *pwd* its expression was higher with EAA treatment than Placebo; conversely 10 *pwd* there was a significant decrease of –R2 expression. TGFβ-R2 levels did not change in Placebo-treated wounds. § *P*<0.05 vs 3 *pwd*. ** *P*<0.01 vs EAA. The data are expressed as mean (± sd) derived from 3 experiments for each treatment and representative images of the blot bands are shown. (D) TGFβ-R1/TGFβ-R2. in EAA-treated wounds 3 *pwd* the ratio was 1:1 (near to sketched line), suggesting that in early phase of wound repair, EAA restrains ECM synthesis. Differently, with Placebo the ratio is approximately 2:1, suggesting the greatly increases of ECM deposition. Ten *pwd*, the TGFβ-R1/TGFβ-R2 ratio increased with EAA treatment, whereas they strongly decreased with the Placebo treatment. The standard deviation bars are not shown because histograms are derived from the ratio between the final average data in B and C.

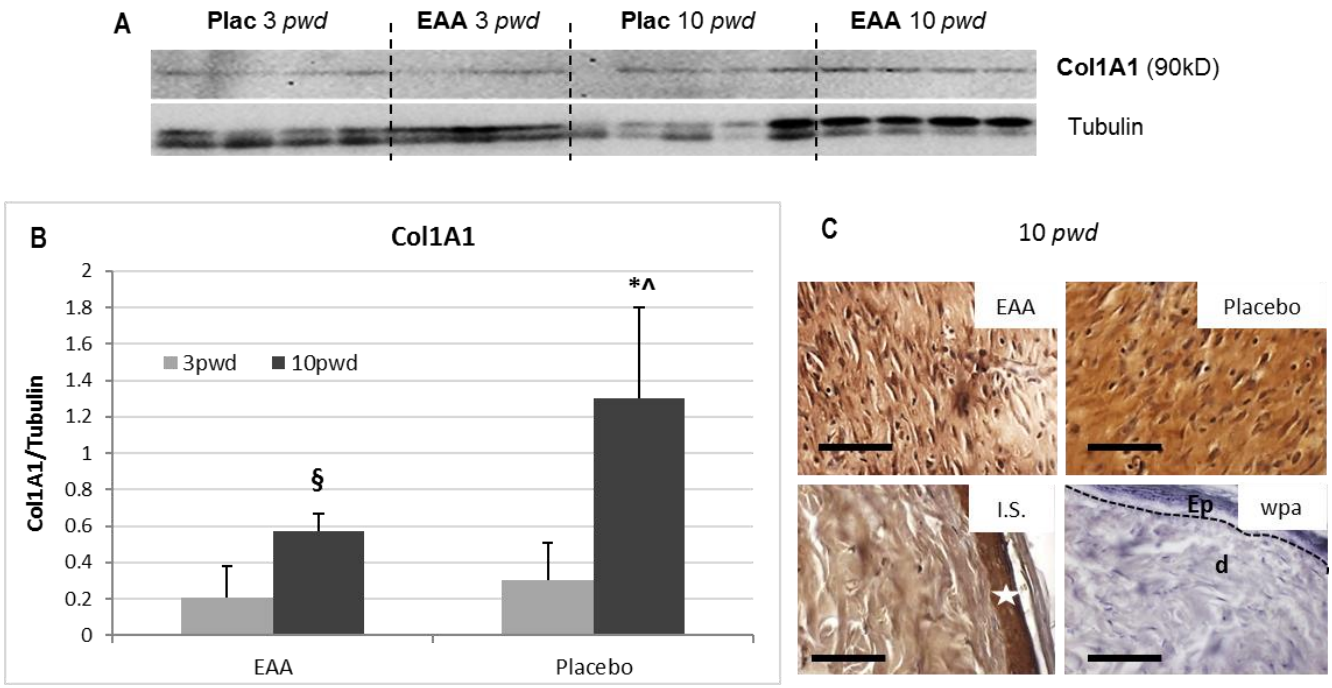

Figure 6. (A) Representative bands of WB using anti-Col1A1 antibody according to treatments and times. (B) WB data shows that EAA-treated and Placebo-treated wounds increased the collagen-1 synthesis mainly 10 *pwd*. However, EAA treatment significantly limited Co1A1 expression than Placebo. Data are expressed as mean (±sd) from 3 experiments for each treatment and representative images of the blot bands are shown. § and ^ *P*<0.01 vs 3 *pwd*; * *P*<0.05 vs EAA. (C) Representative IHC images of anti-Col1A1 10 *pwd*. With EAA treatment, the fibroblasts staining was weak to moderate and uniformly distributed; conversely Placebo-treated wounds had more widespread intense staining. I.S. = intact skin, the staining of dermis was uniformly weak, whereas it was more intense in epidermis (white star). wpa = without primary antibody. Ep = epidermis; d = dermis. Scale bar = 30μm.

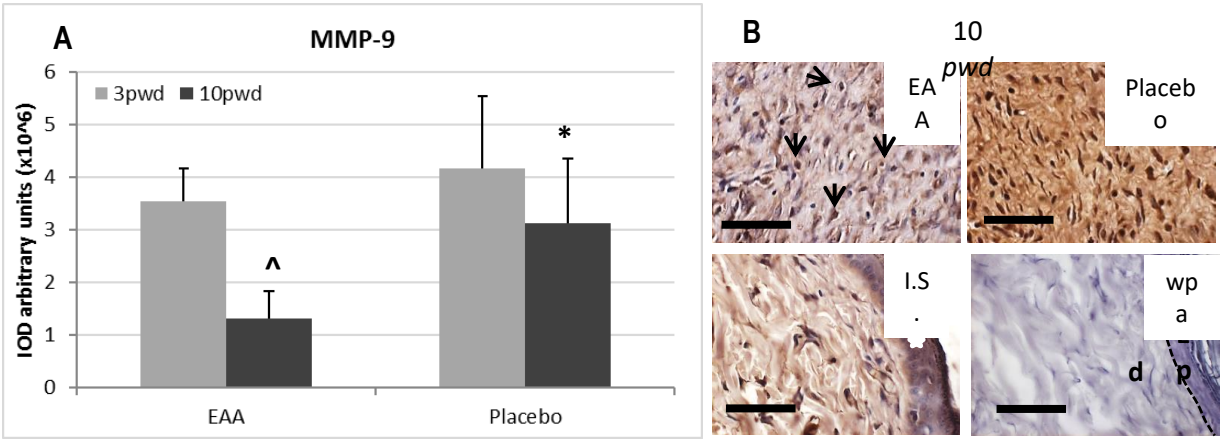

Figure 7. (A) Integrate optical density (mean ±sd) for anti-MMP9 IHC shows that EAA treatment strongly reduced MMP9 staining 10 *pwd*. Placebo-treated wounds did not change the staining intensity. ^ *P*<0.01 vs 3 *pwd*; * *P*<0.05 vs EAA. (B) Representative IHC pictures of anti-MMP9 10 *pwd*. EAA-treated wounds had faint staining of fibroblasts as those of intact skin. However, macrophages (arrows) and fibroblasts had intense reactivity. Placebo-treated wounds had more intense and diffuse staining, furthermore many fibroblasts and macrophages were strongly stained. I.S. = intact skin, the IHC staining was very faint in the dermis and epidermis (white star). wpa = without primary antibody. Ep = epidermis; d = dermis. Scale bar = 30μm.

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