Diet enrichment with a specific essential free amino acid mixture improves healing of undressed wounds in aged rats

Giovanni Corsetti, Claudia Romano, Evasio Pasini, Emanuele Marzetti, Riccardo Calvani, Anna Picca, Vincenzo Flati, Francesco S. Dioguardi

PII: S0531-5565(16)30518-6
DOI: doi: 10.1016/j.exger.2017.06.020
Reference: EXG 10085

To appear in: *Experimental Gerontology*

Received date: 16 November 2016
Revised date: 2 May 2017
Accepted date: 27 June 2017

Please cite this article as: Giovanni Corsetti, Claudia Romano, Evasio Pasini, Emanuele Marzetti, Riccardo Calvani, Anna Picca, Vincenzo Flati, Francesco S. Dioguardi , Diet enrichment with a specific essential free amino acid mixture improves healing of undressed wounds in aged rats, *Experimental Gerontology* (2016), doi: 10.1016/j.exger.2017.06.020

Diet enrichment with a specific essential free amino acid mixture improves healing of undressed wounds in aged rats

Giovanni Corsetti * [1], Claudia Romano [1], Evasio Pasini [2], Emanuele Marzetti [2], Riccardo Calvani [3], Anna Picca [3], Vincenzo Flati [4], and Francesco S. Dioguardi [5]

[1] Division of Human Anatomy and Physiopathology, Department of Clinical and Experimental Sciences, University of Brescia, Brescia, Italy;

[2] Clinical Scientific Institutes "Maugeri", IRCCS. Lumezzane, Cardiac Rehabilitation Division. Lumezzane (Brescia). Italy

[3] Department of Geriatrics, Neurosciences and Orthopedics, Catholic University of the Sacred Heart, Rome, Italy

[4] Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, L'Aquila, Italy;

[5] Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy.

* Corresponding author

Running title:

Essential amino acid intake improves skin wound healing in older rats.

Mailing address:

Dr. Giovanni Corsetti

Division of Human Anatomy and Physiopathology, Department of Clinical and Experimental Sciences. University of Brescia, viale Europa, 11 - 25124 Brescia – Italy

Tel: +39 030 3717484, Fax: +39 030 3717486, E-mail: giovanni.corsetti@unibs.it

Abstract

Chronic wounds are a major, often underestimated, health problem for the elderly. Standard wound care products are not usually manufactured to meet the increased demand of nutrients by skin cells in order to regenerate new tissue and accelerate healing. This work was therefore undertaken to establish whether wound healing could be accelerated by nutritional supplementation with a specific mixture tailored to human need of essential amino acids (EAAs) without topical medication. To this end, using a skin full-thickness excisional model in aged rats, we compared the closure dynamics of undressing wounds in animals fed an EAAs-enriched diet or standard diet. We assessed the degree of fibrosis and inflammation, as well as relevant signaling molecules such as COL1A1, iNOS and TGFβ1. The results showed wound healing was accelerated in EAAs-fed rats, which was accompanied by reduced inflammation and changes in TGFβ1 and COL1A1 expression. Collectively, our findings indicate that dietary supplementation with balanced EAAs diet could serve as a strategy to accelerate wound healing without inducing fibrosis and could therefore be a simple but pivotal therapeutic approach in human also.

Key words. Wound healing, amino acids, skin, aging, rat

Abbreviations list.

COL1A1: collagen type 1 alpha 1

EAAs: Essential amino acids

iNOS: inducible nitric oxide synthase

StD: Standard Diet

TGFβ1: transforming growth factor beta 1

1. Introduction

Chronic skin wounds are a major, often underestimated, clinical and public health problem. In the United States, more than 6.5 million persons suffer from chronic wounds, resulting in US$25 billion spent annually for their treatment (Sen et al., 2009). The ongoing aging of the population, mean that the frequent need for post-surgical and/or traumatic wound care and the increasing incidence of diabetes and obesity are all expected to contribute to a "chronic wound epidemic". The development of new strategies to accelerate wound healing has therefore become a priority, especially in geriatric medicine.

Standard wound care typically involves the use of dressing to protect them from microbial contamination. This promotes healing by creating an aseptic environment that moisturizes the wound tissue and enhances epithelialization and granulation tissue formation. However, wound care products are not usually manufactured to meet the increased demands of nutrients by skin cells needed to regenerate new tissue and close wounds. Previous work from our group showed that the topical application of an amino acids (AAs) mixture (i.e., glycine, proline, leucine, and lysine) and sodium hyaluronate accelerated wound closure and prevented fibrosis in a rat model of excisional wound (Corsetti et al., 2010). Furthermore, Hannessey et al. (1991) demonstrated that nitrogen balance, but not age, impacts collagen production and wound repair in rats.

More recently, long-term supplementation with a specific mixture of a stoichiometrically balanced ratio of essential AAs (EAAs) was shown to prevent senescence-related tissue degeneration of kidney, skeletal muscle and myocardium in rats (Corsetti et al., 2008 and 2010). Interestingly, administration of an essential amino acid, branched-chain AAs-enriched mixture extended the average life span of mice. This was accompanied by enhanced mitochondrial biogenesis and sirtuin 1 expression in cardiac and skeletal muscle (D'Antona et al., 2010). Collectively, these findings suggest that dietary supplementation with specific EAAs mixtures attenuates age-related tissue degeneration.

Based on available evidence, we therefore hypothesize that skin wound healing would be accelerated by nutritional supplementation with EAAs in a stoichiometrically balanced ratio without topical medication. To address this research question, we compared wound closure dynamics and relevant signaling molecules in aged rats fed an EAAs-enriched diet and control rodents on a standard laboratory diet (StD) using a full-thickness excisional model.

2. Materials and methods

2.1. Animals and diet

The experimental protocol was conducted in accordance with the Italian Ministry of Health regulations and complied with the '*The National Animal Protection Guidelines'*. The Ethics Committee for Animal Experiments of the University of Brescia approved the procedures. Thirty-six male Sprague-Dawley rats of 16 months of age (Envigo-Harlan, Italy) were used. The animals were housed in a quiet, temperature- and humidity-controlled room and were kept on a 12/12-h light/dark cycle (lights on from 7 a.m. to 7 p.m.). Rats were randomized 1:1 to receive for 30 days, either an *ad libitum* standard rodent laboratory diet (StD) (Mucedola srl, Milan, Italy) or an iso-caloric and iso-nitrogen diet (produced in accordance with the AIN76-A/NIH-7 rule by Dottori Piccioni, Milan, Italy) containing free EAAs as the only nitrogen source. EAAs were provided in a formula tailored for mammal needs that has been widely used in clinical and experimental studies (Dioguardi, 2008 and 2011; Aquilani et al., 2003; Corsetti et al., 2008; Davidson, 1998). Body weight (Bw), food intake and water consumption were measured every three days. The composition of the two diets is summarized in Table 1.

2.2. Excisional wound model

After 34 days of housing, the rats were surgically inflicted with skin excisional wounds. The cutaneous excisional wound model has proven to be adequate for biochemical and histological assessment of healing (Davidson, 1998) and was performed as described previously (Corsetti et al., 2010). Briefly, each rat was anesthetized with an intra-muscular injection of Zoletil (30 mg/kg Bw) (Virbac, Carros Cedex, France), and the dorsal surface skin was manually shaved and cleaned with 0.1% iodine alcohol.

Four full-thickness round wounds of 5 mm in diameter, two on each side of the dorsal midline, were made with a 5-mm sterile biopsy punch (Kai Industries co. ltd., Oyana, Japan). No

fatalities occurred as a consequence of the surgical procedure. Wounds were left unsutured and uncovered, and the animals were housed individually and allowed to recover (Kapoor et al., 2004).

The wounds of each animal were carefully monitored daily and cleaned as necessary. As described by Ring et al. (2000), the perimeter of each wound was traced onto a glass slide. The wound area (mm^2) was calculated from the wound perimeter using an image analysis program (Image Pro Plus 4.5.1, Images and Computers, Milan, Italy). During the healing time, all wound areas were converted into % compared with the original day when wounds had been made (T0). The surgical wound areas at T0 were considered 100%. None of the rats developed wound infections.

2.3. Sample collection and processing samples for histochemistry

Four animals from each group were euthanized under deep anesthesia at 3, 6, 15 and 30 days post-wounding (pwd). The wounds were quickly excised and bisected at midpoint and post-fixed with immunofix (BioOptica, Milan, Italy) for 24 h at 4°C and processed for paraffin embedding. Wound sections (~5µm thick) were used for histochemical (HC) and immuno-histochemical (IHC) procedures. Five fields of each histological section were analyzed and the average number of inflammatory cells and fibroblasts were calculated. Collagen production was also evaluated by picrosirius stain methods, as previously described (Dayan et al.,1989). Briefly, the sections were de-paraffinized, re-hydrated in distilled water and immersed in 1% phosphomolybdic acid (Sigma-Aldrich, St. Louis, MO, USA) for 5 min and then covered with 0.1% (w/v) Sirius-red F3B (C.I.35780 Science Lab, Huston, TX, USA) in saturated picric acid solution for 1 h at room temperature. The sections were then washed in water and rapidly dehydrated, cleared in xylene and mounted on glass slides. The sections stained with Sirius red were visualized by light microscope (Olympus BX50, Tokyo, Japan) under polarized light obtained with a polarizer filter (Olympus U-ANT, Tokyo, Japan) to analyze first the collagen organization and then fibrosis.

Under these conditions, collagen fibers of varying thickness appeared to be stained to a different degree. During tissue response to injury, fibronectin and type III collagen are synthesized in higher amounts, whereas in normal tissue the major constituent is type I collagen (Williams, et al., 1984). Although the birefringent color is more a measure of collagen fibre size than of collagen type, usually the thick and denser type I collagen fibers are detected as orange to red, whereas the thinner type III collagen fibers appear yellow to green (Koren et al., 2001). The percentage of the collagen area relative to the wound surface was calculated using an image analysis program (Image Pro Plus 4.5.1).

2.4. Immunohistochemistry.

Sections were incubated overnight with polyclonal primary antibodies anti-transforming growth factor beta 1 (TGFβ1; sc-146), anti-collagen type 1 alpha 1 (COL1A1; sc-25974), both from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and anti-inducible nitric oxide synthase (iNOS; ab-3523) from Abcam (Cambridge, MA), diluted 1:100 in PBS. The sections were processed following the manufacturers' instructions, 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. As a negative control, the primary antibody was omitted in the presence of an isotype-matched IgG. To prevent incorrect interpretation of the immunostaining due to the presence of endogenous biotin, experiments using the peroxidase-anti-peroxidase detection system were also carried out and similar results were obtained. Each set of experiments was performed in triplicate, with each replicate carried out under identical experimental conditions.

The staining intensity of IHC slides was evaluated using an optical Olympus BX50 microscope equipped with an image analysis program (Image Pro Plus 4.5.1) and analyzed quantitatively. The optical density (OD) was calculated in marginal and central wound areas by

measuring five fields for each sample. The data were pooled and the mean value was used for the analysis.

2.5. Statistical analysis

Data are expressed as mean ± SD. Differences between groups were assessed by Student's t-test. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1 Bw, food intake and water consumption

Relative to the baseline, the Bw of rats on StD increased during the following 34 days by approximately 5%. In contrast, Bw did not vary for the EAAs-fed animals (Fig. 1A). The Bw of the StD-fed group declined during the pw period, fell below the baseline at 6 pwd and increased thereafter. Animals fed the EAAs diet experienced 1% Bw reduction after 3 pwd, which remained unvaried until 6 pwd and increased thereafter. At 30 pwd, Bw increased by 12.5% and 6% relative to baseline (day 0), in the StD-fed and EAAs-fed groups, respectively (Fig. 1A). The mean daily food intake (g/kg/Bw) was significantly lower in EAAs-fed animals compared to StD-fed (Fig. 1B), while the opposite pattern was observed for water consumption (mL/kg/Bw) (Fig. 1C).

3.2 Wound repair and collagen histochemistry

No signs of wound infection were identified in any animals. The wound repair time was shorter in the EAAs-fed compared with StD-fed animals (Figs. 2A and B). The difference in wound area between EAAs diet and StD was already evident at 3 pwd and peaked at 6 pwd (Fig. 2B). The density of inflammatory cells in the wound area ($n/100\mu^2$) decreased progressively between 3 pwd and 30 pwd in both groups. However, rats EAAs-fed had a significantly lower number of inflammatory cells at each time-point (Fig. 2C). The density of typical fibroblasts with fusiform appearance increased in both groups until 15 pwd, and was significantly higher in EAAs-fed rats. The number of fibroblasts decreased in both groups between 15 and 30 pwd (Fig. 2D).

The organization of collagen fiber bundles in the granulation tissue was evaluated by birefringence patterns of Sirius red-stained sections under polarized light, as described by Rosensteel et al. (2010) (Fig. 3). In both groups, an early production of a network of thin yellow/green bi-refringent collagen fibers corresponding to immature collagen was observed at 3 pwd. Between 6 and 15 pwd, collagen fibers in wounds from StD-fed animals were reduced in

number and thickness relative to the EAAs group (Fig. 3A). In addition, collagen fibers in StD animals, although well oriented, appeared to be rather scattered, whereas they were orderly oriented and packed together in the EAAs group. At 30 pwd, collagen fibers were thick (red bi-refringence), but more disarranged and often massed in the StD fed group (Fig. 3A). In contrast, in EAAs-fed animals collagen fibers were thicker, better oriented and arranged in linear arrays, which was consistent with a more mature connective tissue matrix containing large collagen bundles (Fig. 3A). The mean area of collagen fibers in the wound area according to diets is depicted in Fig. 3B.

3.3. Immunohistochemistry

The modulation of the inflammatory phase is crucial to wound healing. We therefore evaluated the expression of the pro-inflammatory mediator iNOS by IHC. In both groups, the wounds showed intense iNOS staining density at 3 pwd (Fig. 4A). Subsequently, the staining intensity decreased linearly in both diets, but significantly so in EAAs diet (Fig. 4A-C).

iNOS signaling influences collagen production during wound healing (Schwentker, et al., 2003). Indeed, at 3 pwd, COL1A1 immunostaining was low in both groups with a tendency towards higher intensity in the EAAs-fed animals. During the subsequent healing phases, a significantly higher COL1A1 staining intensity was observed in the EAAs-fed group (Fig. 5A-C).

Finally, since collagen synthesis is regulated by TGFβ1, we evaluated the expression of this cytokine. TGFβ1 immunostaining did not show time-dependent differences in either group in the first pw days. However, at 30 pwd, TGFβ1 expression was higher in rats fed with EAAs diet than StD-fed animals (Fig. 6A-C).

4. Discussion

Wound repair is a complex biological process involving multiple biochemical pathways that are sequentially activated and integrated to respond to injury. Various factors, such as wound site and extension, eventual infections, advanced age, overall health status and nutritional status (Gurtner et al., 2008; Cardoso et al., 2004; Martin, 1997), are all major determinants of healing.

An adequate nutritional intake is critical to developing a favorable metabolic environment for wounds (Molnar et al., 2014). Our findings support the relevance of adapting nutrition to wound healing requirements by showing that the administration of a diet containing a balanced EAAs mixture improves and accelerates wound repair, mainly through modulating inflammation and collagen synthesis. Our findings also suggest that inadequate nutritional nitrogen intake would substantially interfere with an efficient healing process (Corsetti et al., 2016).

Collagen is the main protein involved in the repair of injured tissues. In normal tissues, collagen provides strength, integrity and structure. When tissues are disrupted, an increase in collagen production is needed to repair the defect and restore the normal structure and function of the tissue.

Type I collagen, the most abundant collagen isoform, is an integral component of granulation tissue and scar. Type 1 collagen has two α1(I) chains and one α2(I) chain proteins wrapped in a triple helix, which are synthesized under the direction of pro-α1 (Type I, COL1A1) and pro-α2 (Type I, COL1A2) genes. Our findings show that dietary enrichment with EAAs promotes early wound closure, with increased production of collagen fibers and without any signs of fibrosis after 30 pwd. We also observed early, abundant synthesis of COL1A1, suggesting that EAA delivery to the injured area promotes fibroblast activity.

Nitric oxide plays an important role in wound healing. iNOS expression is largely induced after skin injury during the inflammatory and proliferative phases (Reichner et al., 1999) and is essential for wound re-epithelialization (Stallmeyer et al., 1999). Our data show that iNOS

expression in the wound area of EAAs-fed animals is decreased compared with those on StD. This indicates that EAAs may modulate the inflammatory response, contributing to faster wound closure.

Down-regulation of iNOS activity is linked to the expression of cytokines, such as those belonging to the TGFβ super-family (Isenberg et al., 2005). TGFβ1, in particular, is known to be intrinsically involved in wound healing, as it is a key regulator of the production and remodeling of the extracellular matrix through its effect on mesenchymal cells. Indeed, TGFβ1 is fundamental to initiate inflammation, granulation tissue formation and wound contraction by inducing differentiation of fibroblasts into myofibroblasts (Gabbiani, 2003).

Furthermore, TGFβ1 drives collagen synthesis which results in the contraction of the extracellular matrix and subsequent wound closure (Ling et al., 2002; Gabbiani, 2003, Waisman et al., 2010). In addition, TGFβ1 promotes keratinocyte migration during wound closure (Ramirez et al., 2014). The relevance of TGFβ1 signaling for wound healing is reflected by the alteration of wound repair in TGFβ1-deficient mice (Brown et al., 1995) as well as in mice treated with a neutralizing anti-TGFβ1 antibody (Saha et al., 1999). In both these experimental models, impaired late-stage wound repair was observed. Conversely, TGFβ over-expression has been linked to keloid development and formation of hypertrophic scars (Cordeiro, 2002). In agreement with these observations, our results showed that EAAs modulate TGFβ1 production that is lower, even if not significantly, than StD until 15 pwd. Curiously, during the final stage of healing (30 pwd) its expression appear to be higher than the StD. However, this is not dependent by the increase of TGFβ1 with EAAs-diet, but by its strong reduction in StD-fed animals. Thus, the EAAs-diet promotes the adequate TGFβ1 production and regulation during all wound repair phases, reducing the healing time and improving the "quality" of repair. We do not know the reason of these changes so they need further studies. Indeed, it should be considered that the biological role of TGFβ1 in wound healing is very complex and strictly regulated by various conditions at different time (Ramirez et al., 2014).

Potential events which link the EAAs effects on wound healing are depicted in Fig. 7.

4.1. Limitations of the study

The results of this investigation were obtained only through HC and IHC. Although IHC is not suited for accurate quantitative analysis, it offers several advantages. Standard fixation maintains tissue architecture allowing for meaningful morphological assessments. In addition, IHC allows for molecules' localization between different cells types and inside single cells.

5. Conclusions

Unhealed wounds are a major health problem especially for the elderly. Provisional repair, which is often observed in these patients, may reduce protection from environmental insults, with considerable prognostic impact. Our experimental model shows that diet enrichment with EAAs could modulate wound tissue inflammation and collagen deposition accelerating healing without inducing fibrosis in aged animals. These findings could therefore provide the basis for future studies aimed at establishing conditions in which this nutritional supplementation may offer therapeutic advantages for patients suffering from chronic wounds.

Conflict of interest statement

F.S. Dioguardi is the inventor and owner of US patents: N°-US6218420 B1. Compositions based on amino-acids for preventing and treating alimentary overloads in conditions of elevated body nitrogen requirements, without causing calcium losses. N°-US7973077 B2: Amino acid based compositions for the treatment of pathological conditions distinguished by insufficient mitochondrial function and other patents pending on different amino acids based formulations.

The other authors declare no conflicts of interest.

Acknowledgments

The work was supported by grants from Det.Met.Res.Lab. s.r.l. (Milan, Italy). The work was also partly supported by Fondazione Roma (NCDs Call for Proposals 2013) and the "Centro Studi Achille e Linda Lorenzon". The authors would like to thank Dr. Robert Coates (Lecturer and medical writer, *Linguistic Centre of Bocconi University, Milan, Italy*), for his linguistic revision.

References

Aquilani, R., Opasich, C., Verri, M., Boschi, F., Febo, O., Pasini, E., et al., 2003. Is nutritional intake adequate in chronic heart failure patients? J. Am. Coll. Cardiol. 42, 1218-1223.

Ashcroft, G.S., Mills, S.J., Ashworth, J.J., 2002. Ageing and wound healing. Biogerontology. 3, 337-345.

Brown, R.L., Ormsby, I., Doetschman, T.C., Greenhalgh, D.G., 1995. Wound healing in the transforming growth factor-1 deficient mouse. Wound Repair Regen. 3, 25–36.

Cardoso, C.R., Souza, M.A., Ferro, E.A.V., Favoreto, S., Pena, J.D.O., 2004. Influence of topical administration of n-3 and n-6 essential and n-9 nonessential fatty acids on the healing of cutaneous wounds. Wound Repair Regen. 12 (2), 235–243.

Cordeiro, M.F., 2002. Beyond mitomycin: TGF-beta and wound healing. Prog. Retin. Eye Res. 21, 75–89.

Corsetti, G., D'Antona, G., Dioguardi, S.F., Rezzani, R., 2010. Topical application of dressing with amino acids improves cutaneous wound healing in aged rat. Acta Histochem. 112, 497-507.

Corsetti, G., Pasini, E., D'Antona, G., Nisoli E., Flati, V., Assanelli, D., Dioguardi, F.S., Bianchi, R., 2008. Morphometric changes induced by amino acid supplementation in skeletal and cardiac muscles of old mice. Am. J. Cardiol. 101 (11), S26–S34.

DOI: http://dx.doi.org/10.1016/j.amjcard.2008.02.078

Corsetti, G., Pasini, E., Flati, V., Romano, C., Rufo, A., Dioguardi, F.S., 2016. Aging skin. Nourishing form the inside Out, Effects of Good Versus Poor Nitrogen Intake on Skin Health and Healing. In: Textbook of Aging Skin, II Ed. MA. Farage, KW. Miller and HI. Maibach (Eds.), Springer-Verlag Berlin Heidelberg. DOI: 10.1007/978-3-642-27814-3_135-1.

D'Antona, G., Ragni, M., Cardile, A., Tedesco, L., Dossena, M., Bruttini, F., et al., 2010. Branched-chain amino acid supplementation promotes survival and supports cardiac and

skeletal muscle mitochondrial biogenesis in middle-aged mice. Cell Metab. 12 (4), 362-372. doi: 10.1016/j.cmet.2010.08.016.

Dayan, D., Hiss, Y., Hirshberg, A., Bubis, J.J., Wolman, M., 1989. Are the polarization colors of picrosirius red-stained collagen determined only by the diameter of the fibers? Histochemistry. 93, 27–29.

Davidson, J.M., 1998. Animal models for wound repair. Arch. Dermatol. Res. 290 (1), S1–S11.

Dioguardi, F.S., 2011. Clinical use of amino acids as dietary supplement: pros and cons. J. Cachexia Sarcopenia Muscle. 2 (2), 75-80.

Dioguardi, F.S., 2008. Nutrition and skin. Collagen integrity: a dominant role for amino acids. Clin. Dermatol. 26 (6), 636-640.

Gabbiani, G., 2003. The myofibroblast in wound healing and fibrocontractive diseases. J. Pathol. 200, 500–503.

Gouin, J.P., Hantsoo, L., Kiecolt-Glaser, J.K., 2008. Immune dysregulation and chronic stress among older adults: a review. Neuroimmunomodulation 15, 251-259.

Gurtner, G.C., Werner, S., Barrandon, Y., Longaker, M.T., 2008. Wound repair and regeneration. Nature 453, 314-321.

Hennessey, P., Nirgiotis, J.G., Andrassy, R.J., 1991. The effects of age and various fat/carbohydrate ratios on nitrogen retention and wound healing in rats. J. Pediatr. Surg. 26, 367–373.

Kapoor, M., Howard, R., Hall, I., Appleton, I., 2004. Effects of epicatechin gallate on wound healing and scar formation in a full thickness incisional wound healing model in rats. Am. J. Pathol. 165 (1), 299-307. DOI: 10.1016/S0002-9440(10)63297-X

Koren, R., Yaniv, E., Kristt, D., Shvero, J., Veltman, V., Grushko, I., et al., 2001. Capsular collagen staining of follicular thyroid neoplasm by picrosirius red: role in differential diagnosis. Acta. Histochem. 103, 151-157.

Isenberg, J.S., Ridnour, L.A., Espey, M.G., Wink, D.A., Roberts, D.D., 2005. Nitric oxide in wound-healing. Microsurgery 25 (5), 442-451.

Ling, E., Robinson, D.S., 2002. Transforming growth factor-beta1: its anti-inflammatory and pro-fibrotic effects. Clin. Exp. Allergy 32, 175–178.

Martin, P., 1997. Wound healing-aiming for perfect skin regeneration. Science 276 (5309), 75–81.

Molnar, J.A., Underdown, M.J., Clark, W.A., 2014. Nutrition and chronic wounds. Adv. Wound Care (New Rochelle). 3 (11), 663-681. DOI: 10.1089/wound.2014.0530

Prockop, D.J., Kivirikko, K.I., 1995. Collagens: molecular biology, diseases, and potentials for therapy. Ann. Rev. Biochem. 64, 403-434.

Ramirez, H., Patel, S.B., Pastar, I., 2014. The role of TGFb signalling in wound epithelialization. Adv. Wound Care. 3 (7), 482-491. DOI: 10.1089/wound.2013.0466.

Reichner, J.S., Meszaros, A.J., Louis, C.A., Henry, W.L., Mastrofrancesco, B., Martin, B.A., Albina, J.E., 1999. Molecular and metabolic evidence for the restricted expression of iNOS in healing wounds. Am. J. Pathol. 154, 1097-1104.

Ring, B.D., Scully, S., Davis, C.R., Baker, M.B., Cullen, M.J., Pelleymounter, M.A., et al., 2000. Systemically andtopically administered Leptin both accelerate woundhealing in diabetic ob/ob mice. Endocrinology 141, 446–449. DOI: 10.1210/endo.141.1.7373

Rosensteel, S.M., Wilson, R.P., White, S.L., Ehrlich, H.P., 2010. COL1A1 oligodeoxynucleotides decoy: biochemical and morphologic effects in an acute wound repair model. Exp. Mol. Pathol. 89 (3), 307-313. doi: 10.1016/j.yexmp.2010.07.003

Sen, C.K., Gordillo, G.M., Roy, S., Kirsner, R., Lambert, L., Hunt, T.K., Gottrup, F., et al., 2009. Human skin wounds: A major and snowballing threat to public health and the economy. Wound Repair Regener. 17 (6), 763–777. DOI: 10.1111/j.1524-475X.2009.00543.x

Shah, M., Revis, D., Herrick, S., Baillie, R., Thorgeirson, S., Ferguson, M., Roberts, A., 1999. Role of elevated plasma transforming growth factor-beta 1 levels in wound healing. Am J. Pathol. 154**,** 1115–1124.

Stallmeyer, B., Kampfer, H., Kolb, N., Pfeilschifter, J., Frank, S., 1999. The function of nitric oxide in wound repair: inhibition of inducible nitric oxide-synthase severely impairs wound re-epithelialisation. J. Invest. Dermatol. 113, 1090-1098.

Schwentker, A., Billiar, T.R., 2003. Nitric oxide and wound repair. Surg. Clin. North Am. 83 (3), 521–530. DOI: 10.1016/S0039-6109(02)00207-4

Waisman, L.A., Ranjan, R., Roes, J., Krieg, T., Müller, W., Roers, A., Eming, S.A., 2010. Differential roles of macrophages in diverse phases of skin repair. J. Immunol. 184, 3964–3977.

Williams, I.F., McCullagh, K.G., Silver, I.A., 1984. The distribution of types I and III collagen and fibronectin in the healing equine tendon. Connect. Tissue. Res. 12 (3-4), 211-227.

Table 1. Amino acid composition of EAAs and StD diet. * from free AAs only. ° from vegetal and animal proteins and added AAs. bcEAAs = branched chain essential AA; NEAAs = Non-EAAs

	EAAs	**StD**
KCal/Kg	3995	3952
Glucids %	61.76	54.61
Lipids %	6.12	7.5
Nitrogen %	20 *	19.5 °
Proteins: % of total nitrogen content	0	99.58
Free AAs: % of total nitrogen content	100	0.42
AA composition (%)		
L-Isoleucine *(bcEAA)*	18.4	--
L-Leucine *(bcEAA)*	17.4	--
L-Valine *(bcEAA)*	8.8	--
L-Istidyne	18.5	--
L-Lisyne	12.3	1.0
L-Phenylalanine	8.3	--
L-Threonine	6.2	--
L-Cysteine	--	--
L-Cystine	4.0	0.4
L-Tyrosine	3.0	--
L-Methionine	2.5	0.4
L-Triptophan	0.6	0.3
L-Alanine *(NEAA)*	--	--
L-Glycine *(NEAA)*	--	0.9
L- Arginine *(NEAA)*	--	1.1

Figure legends

Figure 1. A. Change in body weight expressed as % of baseline weight. **B.** Daily food intake (g/kg bw). **C.** Daily water consumption (mL/kg bw). * $p<0.05$, ** $p<0.001$

Figure 2. A. Representative dorsal wounds at different times in the two dietary groups. At 15 pwd, the wound closure is complete in rats fed EAAs diet, and the lesion areas are hardly visible at 30 pwd (arrows).

B. Wound area in the two experimental groups. Wound area was set at 100% on the day of wounding (day 0). On subsequent days (3, 6, 15 and 30), the areas are expressed as a percentage of the area on day 0. Measures were collected from 20 wounds at each time-point. * $p<0.05$, ** $p<0.001$

C. Inflammatory cells density ($n/100\mu^2$). The EAAs diet reduced the density of inflammatory cells in the wound area at all time-points. ** $p<0.01$.

D. Fibroblasts density ($n/100\mu^2$). EAAs diet promoted an increase of fibroblast density in the wound area. * $p<0.05$, ** $p<0.001$

Figure 3. A. Sirius red stain (polarized light) of wound area according to diets at different time-points in comparison with uninjured skin. Upper line: StD; Bottom line: EAAs diet. At 3 pwd, collagen fibers were very thin and barely visible in both diets (not shown). The EAAs diet favored the production of a dense network of collagen fibers of greater thickness, especially after 6 and 15 pwd. Scale bar 100 µm.

B. Percentage of collagen fibers area in wounds according to diets. The EAAs diet promoted early collagen fibers production. ** $p<0.01$.

Figure 4. A: The anti-iNOS optical density linearly decreased with time in both diets, but significantly more in EAAs diet. * $p<0.05$, ** $p<0.001$ *vs* StD. **B** and **C:** Representative images of anti-iNOS IHC at 6 pwd in StD- and EAAs-fed animals. Scale bar 100 µm.

Figure 5. A. The anti-COL1A1 optical density increased significantly in EAAs- relative to StD-fed animals. After 3 pwd, the COL1A1 intensity was low in both diets. Wounds of EAAs-fed rats showed greater staining intensity from 6 pwd until 30 pwd. * $p<0.05$, ** $p<0.001$ *vs* StD. **B and C.** Representative images of anti-COL1A1 IHC at 15 pwd from StD- and EAAs-fed animals. Scale bar 50 µm

Figure 6. A. anti-TGFβ1. StD-fed induces early TGFβ1 expression. In contrast, the EAAs diet modulated TGFβ1 production by reducing its production and favoring its expression inside fibroblasts. At 15 pwd, TGFβ1 production was not different between groups, although the EAAs diet showed lower intensity. TGFβ1 staining was significantly higher in EAAs-fed animals only after 30 pwd, but did not change in comparison to 6 and 15 pwd. * $p<0.05$ *vs* StD. **B and C.** Representative images of anti-TGFβ1 IHC at 30 pwd in StD- and EAAs-fed animals. Scale bar 50 µm

Figure 7. Schematic representation of the effects of EAAs intake in wound repair.

Figure 1

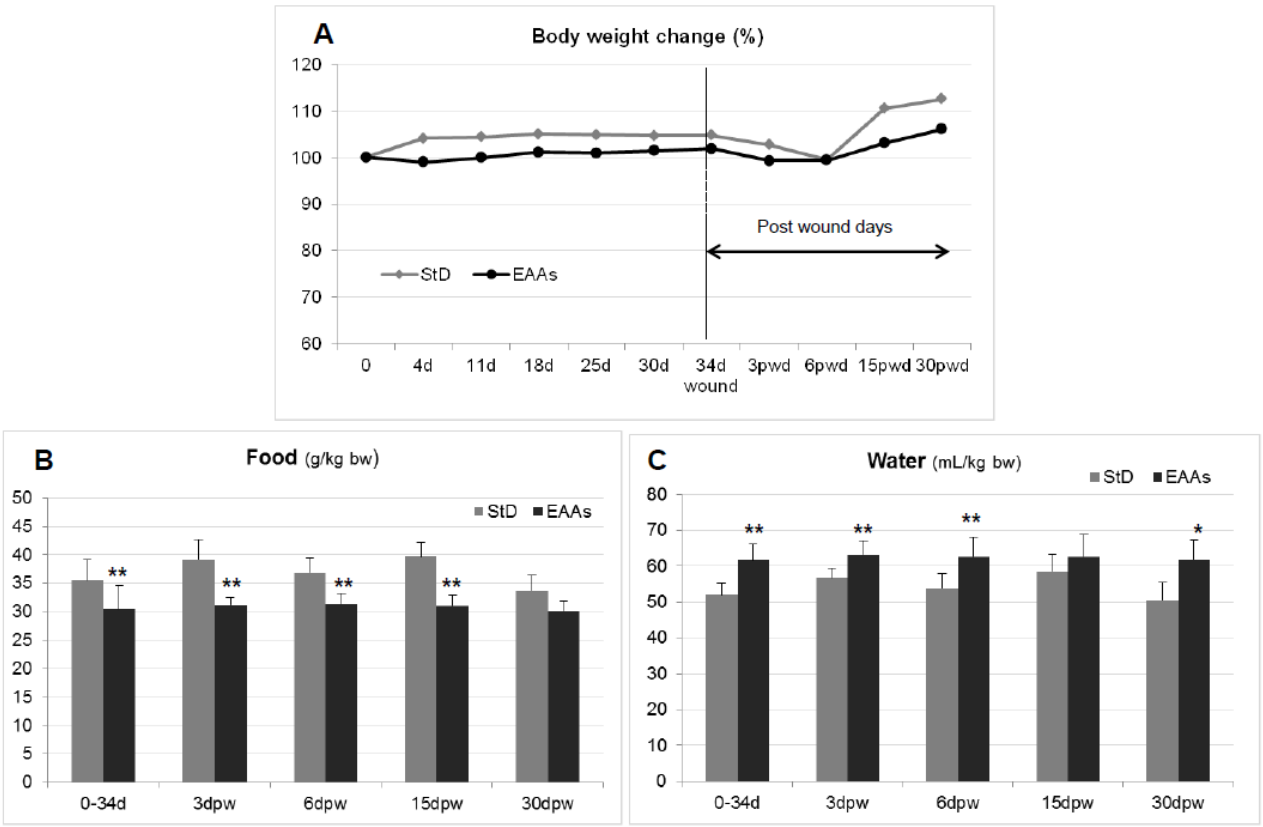


Figure 2

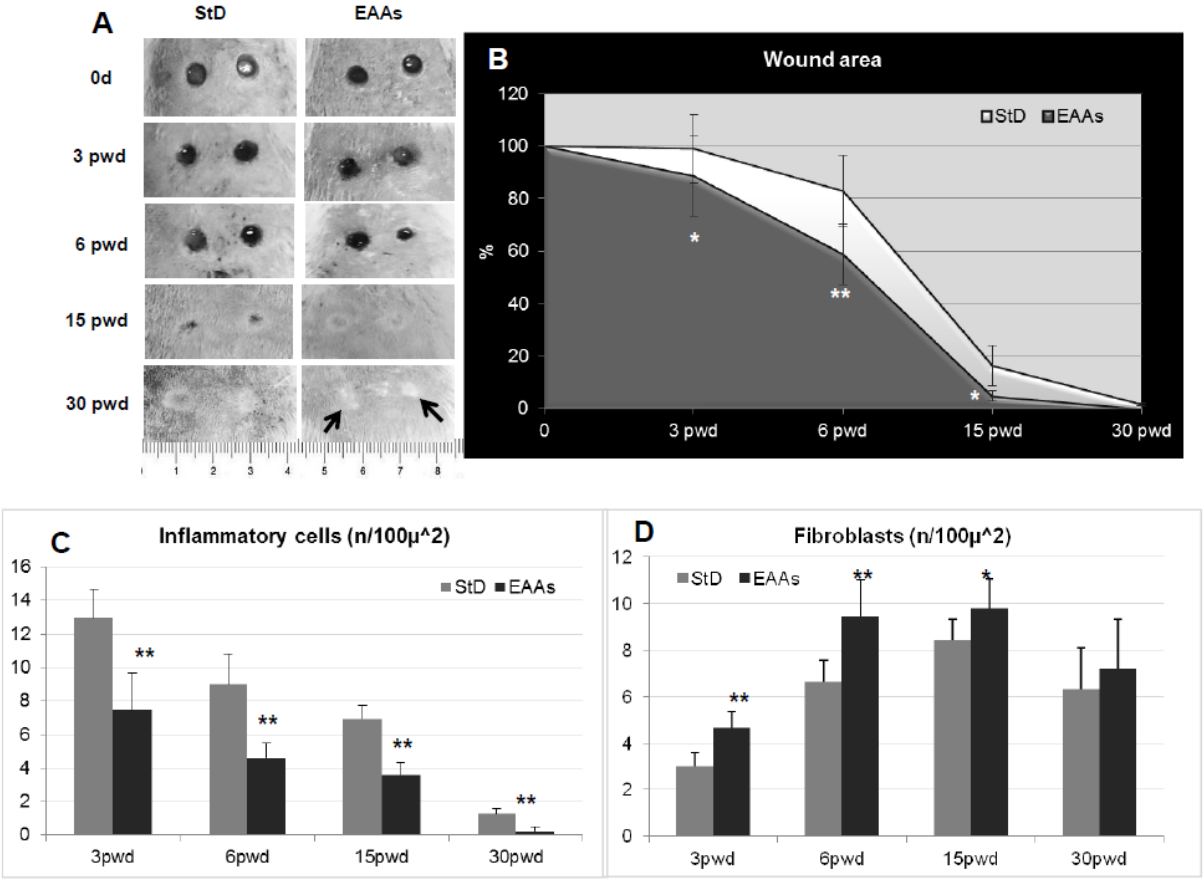


Figure 3

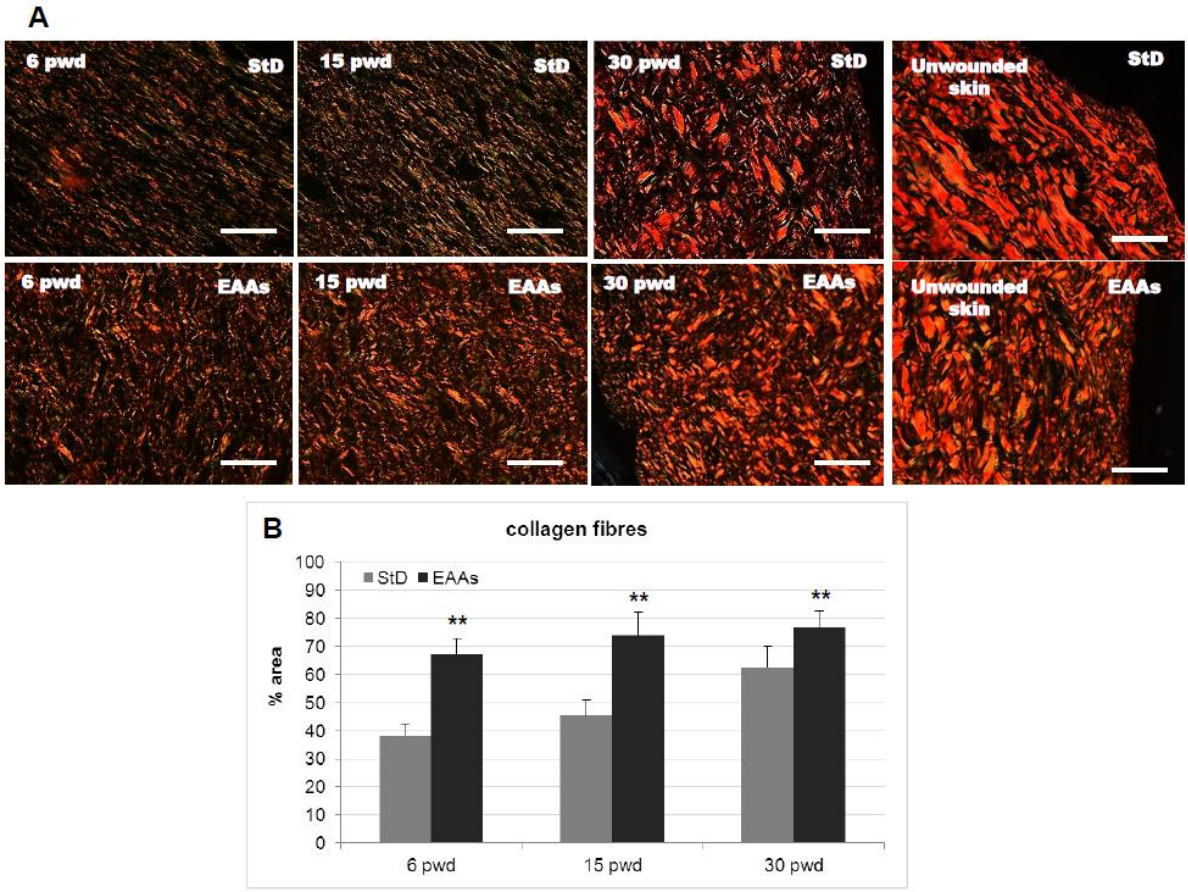


Figure 4

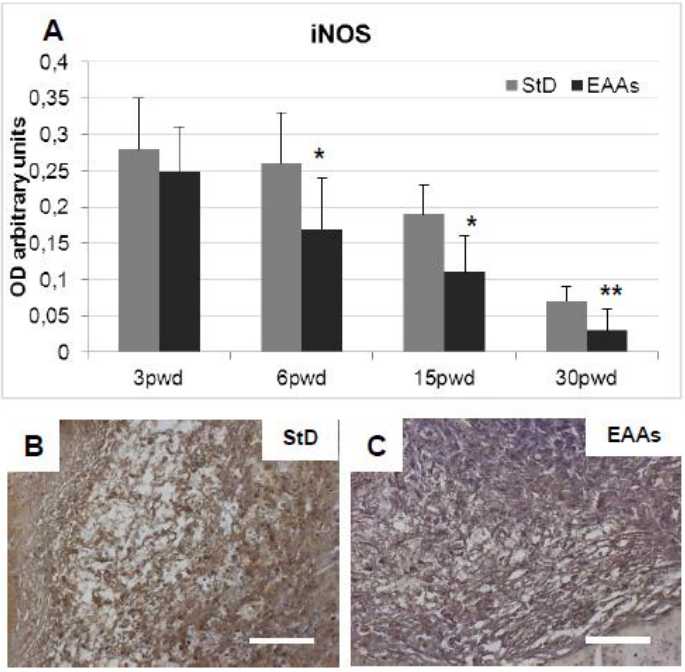


Figure 5

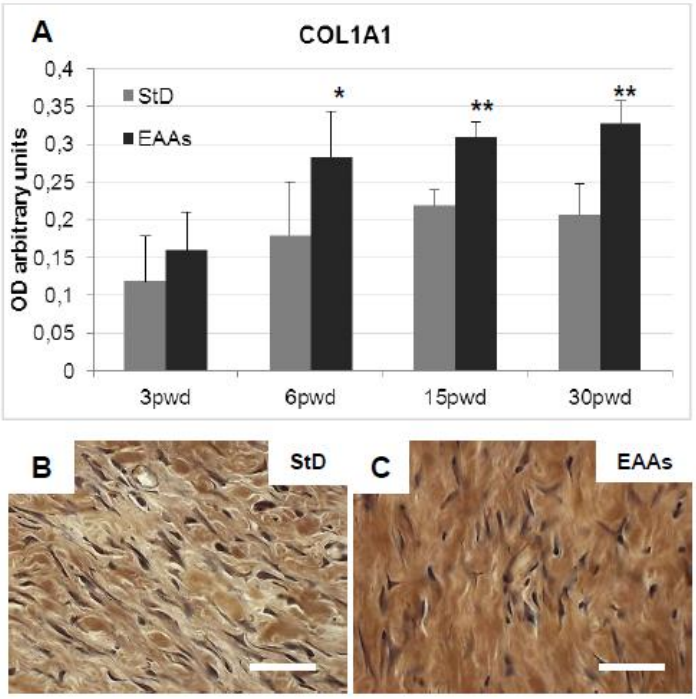


Figure 6

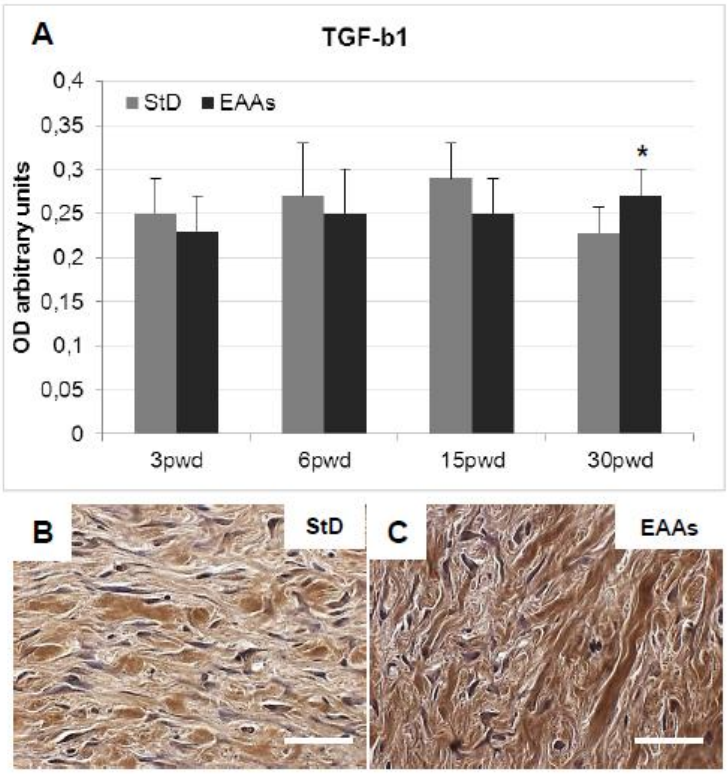
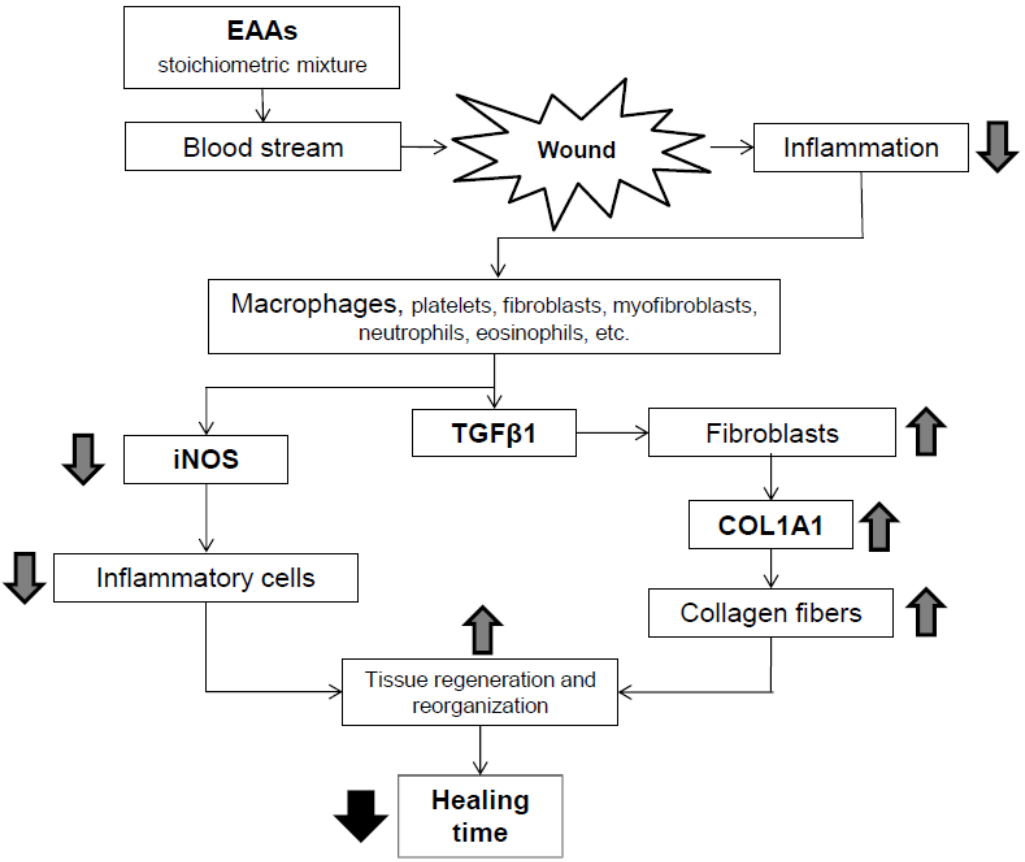


Figure 7



Highlights

- The nutritional status is an important factor in promoting wound healing.
- Topical dressing usually do not provide nutrients to the wound.
- Nutrition with stoichiometric mixture of essential amino acid could promote healing without dressing.
- Nutrition with essential amino acid reduces inflammation, fibrosis and healing time.