Topical application of dressing with amino acids improves cutaneous wound healing in aged rats

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Summary
The principal goal in treating surgical and non-surgical wounds, in particular for aged skin, is the need for rapid closure of the lesion. Cutaneous wound healing processes involve four phases including an inflammatory response with the induction of pro-inflammatory cytokines. If inflammation develops in response to bacterial infection, it can create a problem for wound closure. Reduced inflammation accelerates wound closure with subsequent increased fibroblast function and collagen synthesis. On the contrary, prolonged chronic inflammation results in very limited wound healing. Using histological and immunohistochemical techniques, we investigated the effects of a new wound dressing called Vulnamin\textsuperscript{©} that contains four essential amino acids for collagen and elastin synthesis plus sodium ialuronate (Na-Ial), compared with Na-Ial alone, in closure of experimental cutaneous wounds of aged rats. Our results showed that the application of Vulnamin\textsuperscript{©} dressings modulated the inflammatory response with a reduction in the number of inflammatory cells and inducible nitric oxide synthase (iNOS) immunolocalisation, while increasing endothelial nitric oxide synthase (eNOS) and transforming growth factor-$\beta_1$ (TGF-$\beta_1$) immunolocalisation. Furthermore, the dressing increased the distribution density of fibroblasts and aided the synthesis of thin collagen fibers resulting in a reduction in healing time. The nutritive approach using this new wound dressing can provide an efficacious and safe strategy to accelerate wound healing in elderly subjects, simplifying therapeutic procedures and leading to an improved quality of life.

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Introduction

Treatment of wounds remains an important surgical objective, although chronic wounds caused by various etiologies and/or aging are particularly difficult to treat and often defy closure by both surgical and non-surgical means. Cutaneous healing is a dynamic process that involves a complex interaction of extracellular matrix molecules, resident cells, infiltrating inflammatory cells and soluble mediators. The healing process has four phases; hemostasis, inflammation, tissue regeneration and tissue remodelling with scar formation. These phases overlap in time and duration, each depending on various factors such as tissue type, health status, nutritional status and infections.

Of these phases, the inflammatory response is possibly the most fundamental as it induces production of pro-inflammatory cytokines, such as interferon-γ, interleukin-1β and tumor necrosis factor-α (TNF-α) and is also important in recruiting macrophages, in the proliferation of resident cells and angiogenesis (Schwentker et al., 2002; Eming et al., 2007a, 2007b). Moreover, the lack of apoptotic neutrophils at the wound site deprives the macrophages of their main stimulus to secrete transforming growth factor-β1 (TGF-β1), a key mediator involved in myofibroblast differentiation from fibroblasts (Tomasek et al., 2002; Peters et al., 2005; Hinz et al., 2007). TGF-β1 derived from macrophages also acts as a strong inhibitor of the inducible isoform of nitric oxide synthase (iNOS) during wound healing (Vodovotz et al., 1993). TGF-β1 stimulates fibroblast proliferation and activity producing important extracellular matrix components such as collagen and fibronectin (Roberts et al., 1986; Steed, 1997).

Fibroblasts are the only source of collagen and fibronectin, the main constituent proteins of connective tissue in the dermis, and adequate collagen synthesis is fundamental for wound healing. Like other proteins, collagen synthesis depends totally on the local availability of amino acids found in the final molecule. However, the extremely stringent stoichiometric ratios required for collagen amino acids result from the fact that the single strand collagen polypeptide chain must be folded with another two molecules to form a triple helix precursor molecule of collagen called tropocollagen. Glycine, L-proline and L-lysine are found in extremely regular patterns. The first amino acid is glycine in and nearly 50% of cases the third amino acid is proline. Lysine is inserted in well-defined positions in relation to the glycine and proline, as after assembly of the triple helix, it activates specific hydroxylases to promote hydroxylation of proline and lysine into hydroxyproline and hydroxylysine at specific sites. Lysine and proline hydroxylation generates intermolecular and intramolecular bonds for molecules that give collagen its peculiar flexibility, elasticity and tensile strength. Furthermore, leucine is fundamental, both to promote protein synthesis and to implement elastin synthesis. Elastin is very similar in amino acid composition to collagen, but has a higher content of L-leucine (Dioguardi, 2008).

Healing of dermal lesions requires synthesis and destruction of newly formed collagen at different times. We can presume that the synthesis rate controls the inflammatory cascade connected to the healing process, making synthesis more efficient, with shorter healing time depending on the need for inflammatory mediators (Singer and Clark, 1999).

An important aspect of wound repair is the modulation of nitric oxide (NO) production during the inflammatory phase, because NO regulates collagen formation, cell proliferation and wound contraction in animal models (Witte and Barbul, 2002). NO is a short-lived free radical that is involved in many important biological functions.

NO is produced by nitric oxide synthase (NOS) which exists in three distinct isoforms, two constitutive (endothelial, eNOS and neuronal, nNOS) and one inducible (iNOS). The constitutive isoforms are permanently active, generating low concentrations of NO. The iNOS is induced by a variety of cytokines, growth factors and inflammatory stimuli (Knowles and Moncada, 1999) and is over-expressed during healing, burn injuries, endotoxin exposure, arthritis and inflammatory bowel diseases.

The large amounts of NO formed by iNOS have detrimental effects in inflammatory conditions such as sepsis (Tsukahara et al., 1998). The highest NOS activity occurs during the early phases of wound healing. In rat wound healing, the highest iNOS expression is found between 6 and 24 h, and persists between 1 and 5 days, then slowly decreases over the next 10 days (Albina et al., 1990; Nill et al., 1995; Frank et al., 1998).

It has been shown that most NO synthesis is caused by the inflammatory cells present during early healing phases (Reichner et al., 1999). However many cells, such as fibroblasts, participate in NO synthesis during the proliferative phase after wounding. Therefore, it is believed that iNOS activity can be down-regulated by the resolution of the inflammatory response or by cytokine signalling (Witte and Barbul, 2002). Lack of NO synthesis after blockade of iNOS, or hyper-production of NO from iNOS, has been linked to impaired wound healing. Indeed, most non-healing wounds fail to progress,
remaining in a chronic inflammatory state, generating a cascade of tissue responses that amplify the hostile microenvironment (Lofts et al., 1998; Witte and Barbul, 2002; Schwentker et al., 2002).

Recent studies have demonstrated that cutaneous eNOS expression is significantly increased after excisional wounding in normal mice (Luo et al., 2004). It has been shown that excisional wound closure is delayed by 30% in both eNOS and iNOS knock-out mice compared to wild type mice (Yamasaki et al., 1998; Lee et al., 1999). Furthermore, angiogenesis is a fundamental process of wound repair and eNOS-derived NO plays a pivotal role in this process (Donnini and Ziche, 2002) for pro-angiogenic cytokine activity such as vascular endothelial growth factor (VEGF). However, NO is also involved in VEGF-independent angiogenesis mechanisms that involve monocytes (Leibovich et al., 1994), substance P (Ziche et al., 1994) and TGF-β1 (Roberts et al., 1986). These results indicate that moderate NO production during the inflammatory phase is very important for healing, stimulating fibroblast proliferation, collagen synthesis and angiogenesis.

Our goal was to investigate the mechanism by which a new commercially available wound dressing called Vulnamin® (Professional Dietetics, Milano, Italy) containing four essential amino acids for collagen and elastin synthesis (glycine, L-lysine, L-proline and L-leucine) in an expressly calculated stoichiometric ratio together with sodium ialuronate (Na-Ial), induces wound closure and tissue regeneration.

Cassino and Ricci (2005) showed that Vulnamin® induces rapid tissue regeneration and wound closure in chronic human skin lesions of various etiologies, and concluded that topical treatment may open a new frontier in treatment of patients with chronic ulcers. However, there are no data regarding the biological effects of the treatment at the wound site. We believe that optimal availability of specific amino acids could improve fibroblast activity. Although the thickness of the epidermis probably does not change with age (Whitton and Everall, 1973), structural and functional changes caused by intrinsic ageing occur in the skin of elderly individuals without environmental insults (Fenske and Lober, 1986), indicating that these changes may be due to the alteration of the cellular microenvironment (Ashcroft et al., 1995). Because old rats lose the capability of young rats to efficiently use dietary nutrients, subsequently altering collagen deposition during healing (Hennessey et al., 1991), our study was performed on experimental cutaneous wounds of aged rats.

Materials and methods

Animals

The experimental protocol was approved and conducted in accordance with the Italian Ministry of Health and complied with the ‘The National Animal Protection Guidelines’. Twenty four male Sprague-Dawley rats, aged 26 months, weighing 400±35 g (from Harlan, Italy) were studied. The animals were placed in a quiet room with controlled temperature and humidity. A 12/12 h light–dark cycle was maintained (7 a.m. to 7 p.m.) and they were fed with a standard diet, and had access to water ad libitum.

Each rat was anesthetized with an intramuscular injection of Zoletil (30 mg/kg) (Virbac, Carros Cedex, France), their dorsal surface skin was shaved and cleaned with alcohol and four full thickness wounds, two on each side of the dorsal midline, were made with a 6 mm tissue punch. The depth of wounds was about 2 mm. In each animal, two wounds were dressed with topical applications of Na-Ial only, and two with Vulnamin® (Professional Dietetics, Milan, Italy). Dislodging of the Na-Ial and Vulnamin® dressings was prevented by covering the wounds with gauze fixed with surgical tape. The wound dressing in each animal was monitored and changed twice daily. Following Ring et al. (2000), the perimeter of each wound was traced onto a glass slide. The wound area (mm²) was calculated from the wound perimeter using an image analysis program (Image Pro Plus, Immagini e Computer, Milan, Italy). All wound areas were converted to 100% on the day of wounding (day zero) and on subsequent days, the areas were expressed as a percentage of their original area on day zero. All animals survived the treatment.

Six animals under deep anesthesia were killed and perfused with 10% formalin in phosphate buffered saline (PBS, 0.1 M, pH 7.4) 1, 3, 5 and 10 days after the experimental skin wounding. The wound regions were quickly removed and processed with standard procedure for embedding in paraffin wax.

Histology

Five-μm-thick sections were cut by microtome and stained with hematoxylin and eosin (H&E) using a routine protocol. The inflammatory cells and distribution density of fibroblasts were measured using a bright field optical microscope (Olympus BX50, Tokyo, Japan) in H&E stained sections. The collagen and fibrosis were evaluated by a irius red
staining method (Sweat et al., 1964) using a modified picrosirius procedure as previously described (Dayan et al., 1989). Briefly, the sections were deparaffinized, rehydrated 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. All sections stained with sirius red were analyzed using a light microscope (Olympus BX50, Tokyo, Japan) under normal and polarized light obtained with polarizer filters (Olympus U-ANT, Tokyo, Japan) in order to analyze the initial collagen organization and then the fibrosis. Under these conditions, collagen fibers of different thicknesses are colored differently. During tissue response to injury, fibronectin and type III collagen are synthesized in increased amounts, whereas in normal tissue the major constituent is type I collagen (Williams et al., 1984; Allon et al., 2006). While the birefringent (anisotropic) color is more a measure of collagen fiber 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 (Vranes et al., 1999; Koren et al., 2001; Rizzoni et al., 2005; Buffoli et al., 2005).

**Immunohistochemistry**

Sections were incubated overnight with primary anti-iNOS (NOS2-N20-sc651), anti-eNOS (NOS3-C20-sc654) and anti-TGF-β1 (V-sc146 antibody; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), each diluted 1:50 with PBS, and labelling was visualized using a rabbit avidin biotin complex (ABC) labelling system kit (Santa Cruz), according to the manufacturer’s protocol. They were then dehydrated, cleared in xylene and mounted. The immunohistochemistry control was performed by omitting the primary antibody and incubating instead with isotype matched IgGs. All samples were evaluated using an Olympus BX50 optical microscope equipped with an image analysis program (Image Pro Plus, Immagini e Computer, Milan, Italy) and analyzed quantitatively. The integrated optical density (IOD) was calculated for arbitrary areas, measuring in 10 fields for each sample using a 40 × objective. Data were pooled to show a mean value, and a statistical analysis was applied to compare the results obtained from different experimental groups.

**Statistical analysis**

Morphometric data were expressed as mean ± SD. The statistical significance of the differences between means was assessed by ANOVA followed by Student-Newman-Keuls test. A probability of less than 5% (p < 0.05) was considered significant.

**Results**

Increased healing of Vulnamin®-dressed wounds was time-dependent and differed compared to the Na-lal dressed wounds. Three days post-wounding, healing of the Vulnamin® treated wounds was about 16% greater than that of the Na-lal treated wounds, while after 5 and 10 days post-wounding, the Vulnamin®-treated wound closure was about 30% and 73% greater than that of the Na-lal-treated wounds (Figure 1).

**Vulnamin®**

After three days topical application with Vulnamin®, the mean number of inflammatory cells in the wound area decreased by about 43% (1.3 ± 0.15 cells/1000 μm² vs 2.3 ± 0.18 on the first day). This reduction was more evident after five days post-wounding (0.54 ± 0.12 cells/1000 μm²), also close to the border of the wound, whereas unwounded control skin had no inflammatory cells (Figure 2). The density of fibroblasts had already increased the first day after wounding (1.21 ± 0.18 cells/1000 μm²) and was very high (136%) after three days (2.86 ± 0.16 vs 1.21 ± 0.18 of the first day), about three fold (3.01 ± 0.17 cells/1000 μm²)

![Figure 1. Change in wound healing area on different days expressed as a percentage of area on day zero. Dense dots = Vulnamin dressed; sparse dots = Na-lal dressed.](image-url)
and about five fold ($5.0 \pm 0.36$ cells/1000$\mu$m$^2$), respectively, after three and ten days after wounding (Figure 3). At the same time, we observed under polarized light optics the production of a network of thin orange/yellow to yellow/green collagen fibers from three days post-wounding (Figure 4). The dense network of thin collagen fibers indicated new deposition of collagen that was not present in unwounded control skin. The percentage of collagen fibers was statistically evaluated and is shown in Figure 5.

iNOS immunolabelling decreased strongly in fibroblasts during the first three days post-wounding. After this, it decreased slowly and was similar to...
controls (Figure 6). The statistical analysis of iNOS immunolabelling confirmed the qualitative results obtained by this morphological study, indicating a rapid decrease from three days after the lesion (from 30 to 15 IOD) and a slow decrease from three to ten days (from 15 to 5 IOD), as shown in Figure 7.

We observed a slight increase in eNOS immunolabelling, from three days post-wounding in fibroblasts, and this was strongly detected after five days (Figure 8). These results were confirmed by quantitative analysis and are shown in Figure 9. The last marker that we studied, TGF-β1, increased strongly by three days and then slowly decreased (Figure 10); its quantitative analysis is shown in Figure 11.

### Na-laluronate

With topical application of Na-lal the parameters changed more slowly. After three days post-lesion the number of inflammatory cells decreased by only about 5% (3.03 ± 0.28 cells/1000 μm² vs 3.2 ± 0.23 of the first day). Five and ten days after wounding, the inflammatory cells decreased, respectively, by about 26% (2.36 ± 0.3 cells/1000 μm²) and 63% (1.19 ± 0.18 cells/1000 μm²) (Figures 2, 3). According to these data, the fibroblast density increased slowly after one day post-lesion (0.5 ± 0.15 cells/1000 μm²) and about one-fold after three days (0.9 ± 0.17 cells/1000 μm²), about three-fold five days after lesion (1.91 ± 0.21 cells/1000 μm²) and about seven-fold ten days after lesion (3.5 ± 0.36 cells/1000 μm²). However, the number of fibroblasts was always lower than in the Vulnamin-treated wounds (Figure 3). In addition, we observed very low production of thin yellow to green collagen fibers from three days post-wounding (Figure 4). After five and ten days post-lesion, the collagen production of thin fibers increased moderately. The percentage of collagen fibers was statistically evaluated and is shown in Figure 5.

iNOS was very strongly detected in fibroblasts after one day and immunolabelling decreased moderately after three days (from 42 to 34 IOD). Between the third and fifth days, the iNOS immunolabelling did not vary significantly and then remained strong (Figure 6). iNOS immunolabelling decreased slowly after ten days (from 33 to 18 IOD) as shown in Figure 7.

eNOS immunolabelling increased faintly after three days post-lesion, whereas it increased moderately after five days, mainly in fibroblasts. The control group showed a faint immunolabelling of eNOS only in endothelial cells (Figure 8). The quantitative analysis of eNOS immunolabelling according to time after wounding is shown in Figure 9.

TGF-β1 immunolabelling faintly increased after three days (Figure 10), moderately after the fifth day and strongly only after ten days post-lesion. Its quantitative analysis is shown in Figure 11.
Discussion

The main finding of our study is that Vulnamin® dressings accelerate wound healing in experimental skin lesions with modulation of the inflammatory phase, reduction of iNOS and increase in TGF-β1 and eNOS immunolabelling.

Successful repair after tissue injury requires the resolution of the inflammatory response. Most non-healing wounds fail to progress through the normal phases of wound repair, remaining in a chronic inflammation state (Loots et al., 1998). Of the different phases of healing, the inflammatory phase acts as a limiting factor. Because inflammation is important in counteracting possible bacterial infection, this can create a problem for wound closure. Immediately after injury, clotting blood creates a hemostatic plug in which platelets and leukocytes are trapped and aggregated and these release a wide variety of factors that amplify the aggregation response (Szpaderka et al., 2003). Within a few hours post-injury, the neutrophils in the wound, activated by pro-inflammatory cytokines such as TNF-α, IL1 and IFN-γ, transmigrate across the endothelial cell wall of capillaries, leading to the expression of various classes of adhesion molecules fundamental for leukocyte adhesion and diapedesis. Chemoattractant cytokines initiate post-wounding inflammation and NO modulates these cytokines.

iNOS is upregulated following tissue injury, perhaps as part of a primordial anti-microbial mechanism (Bogdan et al., 2000) and considerable evidence indicates that increased NO production is beneficial to normal healing (Efron et al., 2000). In a rat dermal wound model, iNOS was demonstrated in infiltrating macrophages within 6–24 h of injury, slowly decreasing over the subsequent 10 days (Albina et al., 1980).

In large doses, NO has been shown to be cytostatic to many cell types, including endothelial cells, smooth muscle cells, hepatocytes and fibroblasts (Garg and Hassid, 1990; Stadler et al., 1991; Thomae et al., 1995). However, low concentrations of NO can stimulate cell proliferation (Efron et al., 1991; Du et al., 1997). Collagen synthesis correlates with NO synthesis during wound healing. Inhibition of iNOS by competitive inhibitors decreases collagen deposition, whereas moderate NO administration enhances matrix synthesis (Schaffer et al., 1996, 1997, 1999; Murrel et al., 1997; Witte et al., 1997; Thornton et al., 1998). Our data show that cutaneous wounds dressed with Vulnamin® quickly decreased, but did not inhibit iNOS, and this modulation correlated with fewer inflammatory infiltrates and reduced inflammation. The low level of iNOS is fundamental to help proliferation of resident cells and recruitment of macrophages. Furthermore, the moderate presence of inflammatory cells stimulates macrophages to secrete TGF-β1 and also stimulates fibroblast activity.

Recent findings show that there is significantly increased cutaneous eNOS levels as well as constitutive NOS enzymatic activity after excisional wounding in normal mice (Luo et al., 2004). We also
observed that wounds treated with Vulnamin® greatly increased eNOS immunopositivity by five days post-lesion when the inflammatory phase had finished. The high levels of eNOS indicate a beneficial effect of neo-angiogenesis in new tissues in the wound areas, accelerating and improving the final phases of wound closure and re-epithelialization. These results agree with an earlier report showing that impaired wound healing was paralleled by decreased wound eNOS expression (Stallmayer et al., 2002).

TGF-β1 is a potent chemoattractant, mainly produced by macrophages at the wound site, and it is also one of the strongest iNOS inhibitors during wound healing (Vodovotz et al., 1993). TGF-β family proteins are known to stimulate collagen and fibronectin formation in a variety of fibroblast cell lines (Roberts et al., 1986; Steed, 1997). Therefore, the fast and consistent increase of TGF-β1 immunolabelling after the Vulnamin dressing could also be a sign of macrophage activation that plays a fundamental role in reducing inflammation and increasing fibroblast activity and collagen production. This may indicate that decreased iNOS expression and modulation of NO synthesis are related to enhanced expression of TGF-β1. Presumably, iNOS activity can be downregulated by the over-expression of TGF-β1, with the consequent resolution of the inflammatory phase. The high expression of TGF-β1 could stimulate the fibroblasts to produce a dense collagen network. In addition, fibroblast activity could be facilitated by reduced inflammation in the wound area due to decreased iNOS expression and neutrophil infiltration. In Vulnamin® dressed wounds, the slow decrease of TGF-β1 immunolabelling three days post-lesion, may also have improved the final phase of wound closure. Both keloids and hypertrophic scars express aberrantly elevated levels of TGF-β1 (Ladin et al., 1995). So decreased TGF-β1 in the final phase of wound repair could reduce scar formation, thus improving normal wound closure. The possible mechanism of Vulnamin® in wound closure is summarized in Figure 12.

In humans, protein energy malnutrition (PEM) and involuntary weight loss is a common problem in the elderly population. In elderly burn patients, PEM has been diagnosed in 61% of patients. There is a significant increase in infection rate, decrease in the healing rate of a standard skin graft donor site and increased hospitalisation in the PEM group compared with well-nourished elderly burn patients. Mortality with PEM is significantly higher (17%) compared with 9% without PEM (Demling, 2005). Because PEM alters the general, but also local, availability of amino acids, the chance of rapidly providing the correct ratio of amino acids suitable to promote efficient healing by collagen synthesis directly in the lesion area may provide an easy therapeutic treatment for these frail patients.

Our data show that cutaneous wounds dressed with topical application of Vulnamin® modulate...
the inflammatory response, stimulate the activation and proliferation of fibroblasts rapidly producing a collagen fiber network, with a consequently shorter healing time. This nutritive approach applied to wound dressings could provide an efficacious and safe strategy to accelerate cutaneous wound healing in old patients, simplifying therapeutic procedures and improve the quality of life.

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Disclosure of interests

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Wound closure with amino acids dressing


