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Original Paper

Modulation of MCP-1, TGF-β1, and α-SMA Expressions in Granulation Tissue of Cutaneous Wounds Treated with Local Vitamin B Complex: An Experimental Study

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Key Words
Granulation tissue · Vitamin B complex · TGF-β1 · MCP-1 · α-SMA

Abstract
Vitamin B complex can modulate the inflammatory response and activate wound healing. However, the action mechanisms involved in this process are still unclear. The aim of this study was to evaluate the effects of vitamin B complex on the modulation of monocyte chemotactic protein (MCP)-1, transforming growth factor (TGF)-β1, and α-smooth muscle actin (α-SMA) in granulation tissue growth. Cutaneous ulcers on Wistar rats were topically treated with vitamin B complex. MCP-1, TGF-β1, and α-SMA expressions were evaluated 24, 72, and 168 h after the treatment. Inflammatory cells were counted and collagen fibril staining was performed. After 24 h, more mononuclear cells ($p \leq 0.01$) and a higher MCP-1 ($p \leq 0.05$) and TGF-β1 ($p \leq 0.01$) expression were observed. After 72 h, the number of fibroblasts and mononuclear cells ($p \leq 0.05$) was elevated. After 168 h, an increased number of fibroblasts, myofibroblasts, and blood vessels ($p \leq 0.01$) as well as a strong intensity of collagen fibril staining were seen. At that point, the cells presented a higher TGF-β1 expression ($p \leq 0.05$), and the size of the ulcer area was decreased ($p \leq 0.01$). We can conclude that vitamin B complex may stimulate a positive modulation of MCP-1, TGF-β1, and α-SMA expressions in granulation tissue of cutaneous ulcers.

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Introduction

Cutaneous wound healing is a dynamic process involving 3 stages: (1) inflammation, (2) new tissue formation (e.g. fibroplasia, neovascularization, or re-epithelialization), and (3) tissue reorganization or remodeling of the extracellular matrix [1, 2]. After an injury, the damaged blood vessels, degranulated platelets, and parenchymal cells secrete several mediators of wound healing including transforming growth factor (TGF)-β and monocyte chemotactic protein (MCP)-1. These substances do not only recruit inflammatory leukocytes (e.g. neutrophils or monocytes/macrophages) to the injury site, but they also contribute to the formation of granulation tissue and the contraction of the wound. The granulation tissue which is responsible for wound healing consists of new blood vessels, fibroblasts, inflammatory cells, myofibroblasts, and a provisional extracellular matrix [1, 2]. With different stimuli (e.g. TGF-β or mechanical tension), fibroblasts can express α-smooth muscle actin (α-SMA) and form myofibroblasts with a contraction capacity [2–4].

Vitamins, which are considered dietary substances required for a normal cellular metabolism, act as coenzymes and can also be significant in wound healing [5]. For example, the vitamins of the B complex group may modulate inflammation [6–8] and activate healing [9–20]. Furthermore, in cases of acute pain, the addition of B vitamins to diclofenac can increase its analgesic effect [21]. However, the action mechanisms of vitamin B complex in cutaneous wound healing still remain unclear. In the present study, we investigated the role of vitamin B complex in the modulation of MCP-1, TGF-β1, and α-SMA expressions in granulation tissue of cutaneous wounds.

Materials and Methods

Thirty male Wistar rats (56 days old) were divided into 2 groups. The rats (n = 15) in the control group received no treatment. In the experimental group, the wounds of the rats (n = 15) were treated with vitamin B complex. This study was approved by the Ethics Committee for Animal Usage (CEUA) of the University of São Paulo – USP, Brazil (protocol No. 04.1.700.53.9).

After injecting an intramuscular general anesthesia using 0.75 ml compazine and 0.038 ml ketamine, the rats' dorsal regions were depilated. A skin excision of 6 × 6 mm was performed on the rats' subscapular regions using a punch instrument that contained a stop resin at 2 mm depth. In the experimental group, the rats' ulcers were topically treated with a single dose of vitamin B complex (5 mg vitamin B1, 2 mg vitamin B2, 3 mg vitamin B5, 2 mg vitamin B6, 20 mg nicotinamide, and 0.25 mg biotin; Bayer). The size of the wound was measured daily. The ulcer area and the percentage of contraction were then calculated. The composition and the concentration of vitamin B complex were analyzed using high-performance liquid chromatography.

At 24, 72, and 168 h after the treatment, skin samples were harvested and fixed in 4% paraformaldehyde. The samples were cut into 4-μm-thick sections and stained with hematoxylin and eosin and Masson trichrome. Immunohistochemical techniques were used for the analysis of MCP-1, TGF-β1, and α-SMA expression.

Immunohistochemistry

Histological sections were placed on organosilane-pretreated slides, deparaffinized with xylene, rehydrated, and incubated with 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. For different cells quantification, the slides were incubated with primary

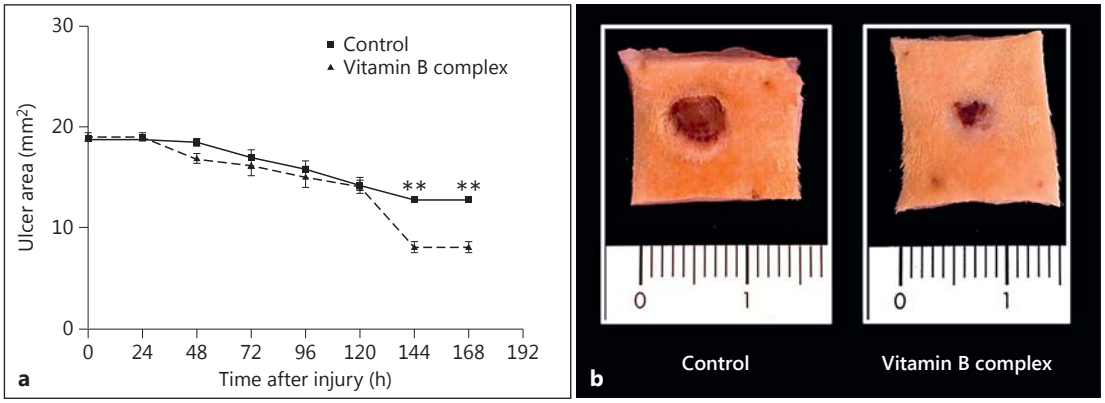

Fig. 1. a Wound area analysis at different points in time. **b** Clinical aspects of the cutaneous ulcers at 168 h after the injury. ** $p \leq 0.01$.

antibodies for 2 h: monoclonal mouse anti-human α-SMA antibody (1A4 clone, code M0851, 1:250 dilution, Dako), polyclonal goat anti-rat MCP-1 antibody (code R17, 1:100 dilution, Santa Cruz Biotechnology Inc.), or polyclonal goat anti-human TGF-β1 antibody (code 6G, 1:300 dilution, Santa Cruz Biotechnology Inc.). Subsequently, the sections were incubated with a second biotin antibody (Universal Kit, Novocastra Laboratories Ltd.) for 15 min. Then, the histological sections were developed using 3,3′-diaminobenzidine (Sigma Chemical Co.) as a chromogen and light counterstaining with Meyer hematoxylin. The slides were dehydrated in graded alcohol, cleared in xylene, and mounted in Permount (Merck, Darmstadt, Germany). We also prepared negative controls, omitting the primary antibody from the assay and replacing it with nonimmune goat serum for MCP-1 and TGF-β1 and nonimmune mouse serum for α-SMA.

Cellular Quantification

The quantity of cells was evaluated histologically in tissues stained with hematoxylin and eosin. The blood vessels were quantified using the immunohistochemical staining for α-SMA. Six random microscopic fields per wound (2 fields at the base and 2 fields on each edge) were selected using a microscope with a 20-point ocular graticule (catalog No. 474004, Zeiss) with ×400 magnification, covering a total of 0.345 mm^2 per wound.

Statistical Analysis

The analysis of variance, followed by a Bonferroni test, was applied in order to quantitatively compare the presence of different cells as well as the immunohistochemical expression of TGF-β1, MCP-1, and α-SMA at different times and for different treatments. A probability of ≤ 0.05 was considered significant.

Results

Clinical Analysis

A decrease in the size of the wounded area could be observed after 120 h (day 5; fig. 1a). Those ulcers that had been treated with local vitamin B complex presented significantly higher contractions ($p \leq 0.01$) 144 and 168 h after the injury (55 and 58%, respectively)

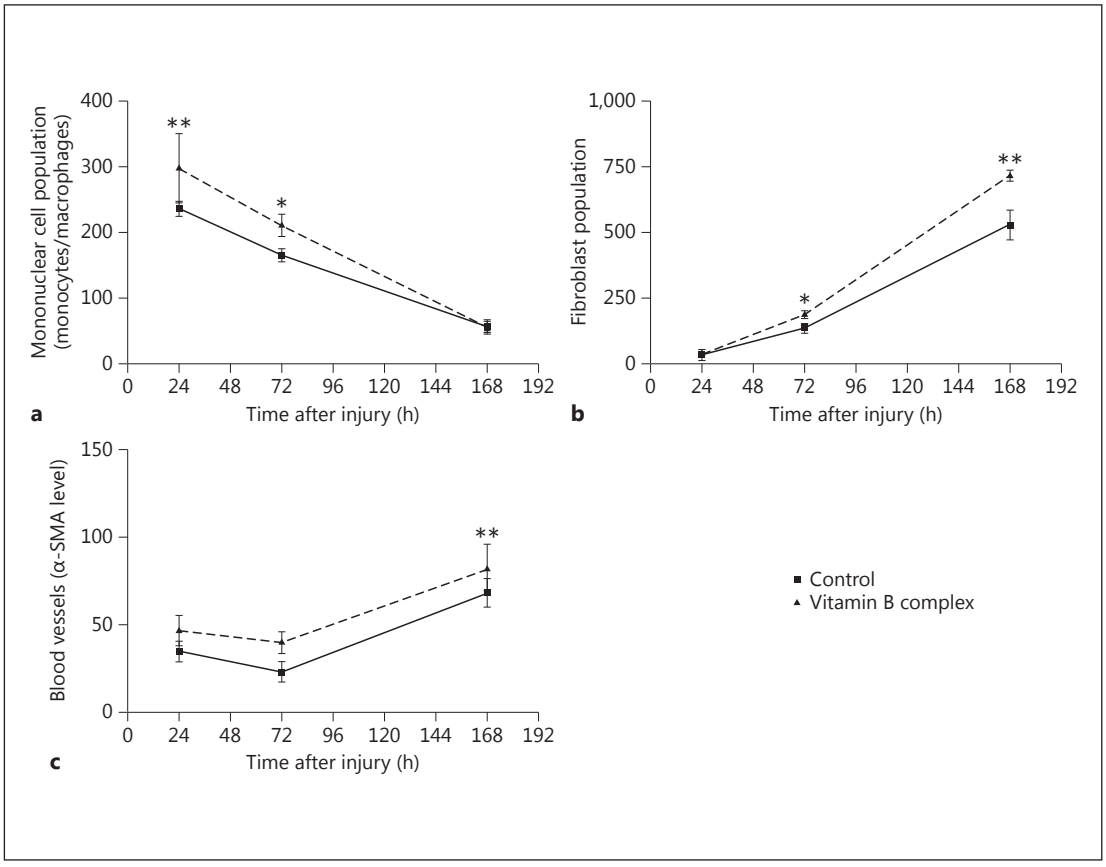

Fig. 2. Quantification of mononuclear cells (monocytes/macrophages; **a**), fibroblasts (**b**), and blood vessels (**c**) using hematoxylin and eosin staining (**a**, **b**) or immunohistochemical staining for α-SMA (**c**). * $p \leq 0.05$; ** $p \leq 0.01$.

when compared to the ulcers of the control group, which decreased only by 30 and 32.1%, respectively (fig. 1a). The clinical aspects of wound contractions are shown in figure 1b for samples taken 168 h after the injury.

Histological Analysis

At 24 h after the injury, although the skin wounds of both the control group and the experimental group had filled with fibrin and showed a dehydrated crust on the surface, the rats in the experimental group presented less edema and fewer neutrophils (data not shown) as well as a statistically larger mononuclear inflammatory cell population (monocytes/macrophages; $p \leq 0.01$; fig. 2a) mainly in the middle and deep regions. Moreover, 72 h after the injury, those ulcers treated with local vitamin B complex presented more fibroblasts ($p \leq 0.05$; fig. 2b) and mononuclear cells (monocytes/macrophages; $p \leq 0.05$; fig. 2a) than the ulcers from the control group. At 168 h after the injury, the ulcers of both groups (control and experimental) were filled with granulation tissue (fig. 3a, b). However, the numbers of fibroblasts ($p \leq 0.01$; fig. 2b) and blood vessels ($p \leq 0.01$; fig. 2c) were significantly higher in the experimental group than in the control group. In addition, more collagenization was observed in the experimental group with Masson trichrome stain mainly in the middle and deep regions (fig. 3c, d).

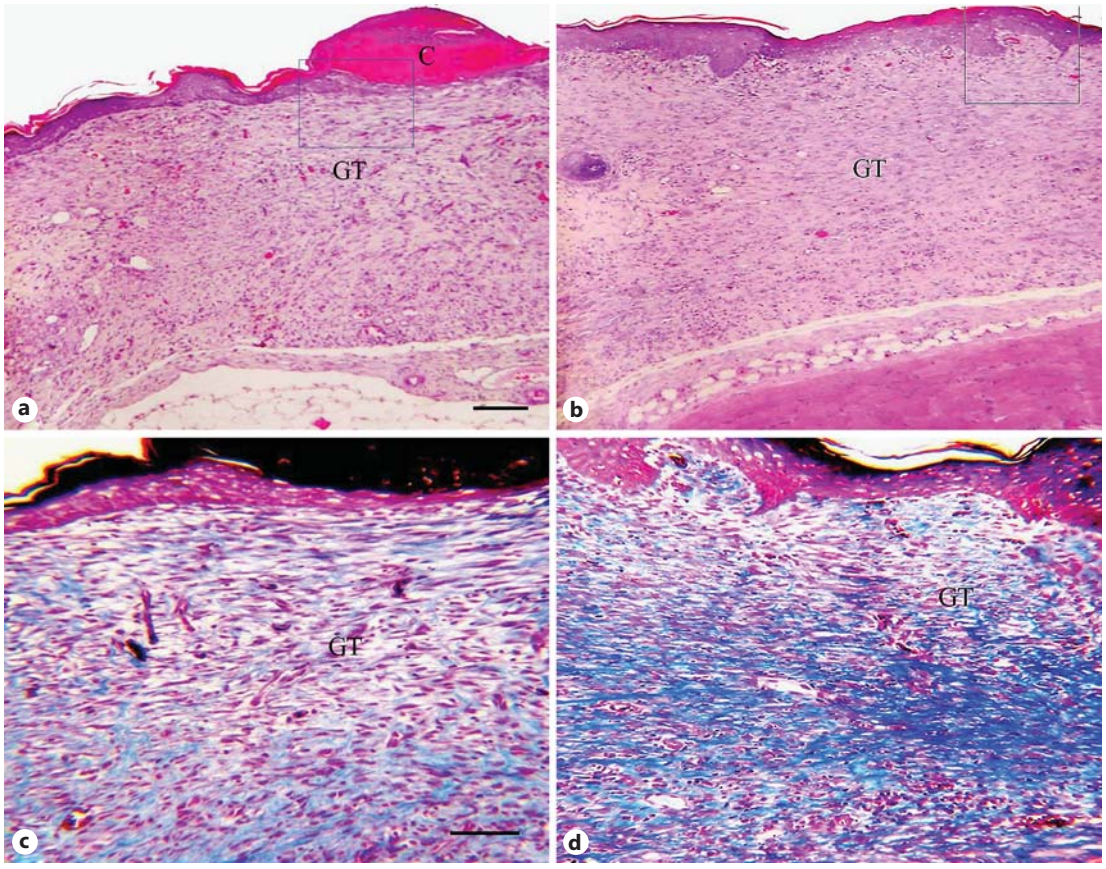

Fig. 3. Histological analysis of the cutaneous ulcers stained with hematoxylin and eosin (**a**, **b**) and Masson's trichrome (**c**, **d**) at 168 h after the injury. The experimental group (**b**, **d**) presented a more developed granulation tissue showing more collagenization on Masson's trichrome stain mainly in the middle and deep regions when compared to the control group (**a**, **c**). C = Crust; GT = granulation tissue. Scale bar = 1 mm (**a**, **b**); scale bar = 0.1 mm (**c**, **d**).

Immunohistochemical Analysis

Analysis of MCP-1 Expression

At 24 h after the injury, MCP-1 was predominantly expressed in mononuclear inflammatory cells (monocytes/macrophages), fibroblasts, and endothelial cells (fig. 4b) in both groups. The peak in MCP-1 expression occurred 24 h after the injury; however, it was significantly higher in the experimental group than in the control group ($p \leq 0.05$; fig. 4a).

Analysis of TGF-β1 Expression

At 24 h after the injury, TGF-β1 expression was mainly verified in mononuclear inflammatory cells (monocytes/macrophages), fibroblasts, and endothelial cells in both the control and the experimental group (fig. 4d). In contrast to the control group, which presented a constant TGF-β1 expression at all times (after 24, 72, and 168 h), the ulcers of the rats treated with vitamin B complex presented two peaks in TGF-β1 expression. One of these peaks took place 24 h after the treatment, whereas the other peak occurred 168 h after the treatment. At both points in time, the TGF-β1 expression in the experimental group was significantly higher (24 h, $p \leq 0.01$; 168 h, $p \leq 0.05$) than that in the control group (fig. 4c). At 168 h after the injury, TGF-β1 was also expressed in epithelial cells (data not shown).

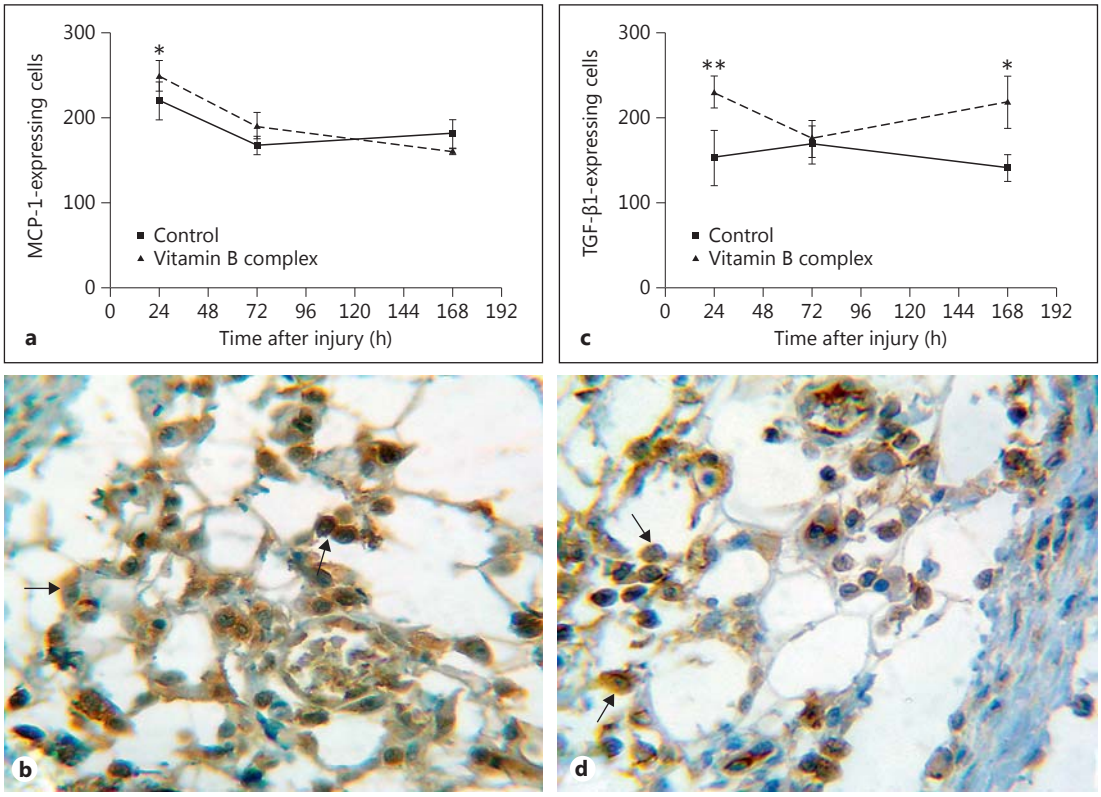

Fig. 4. Quantification of MCP-1- (**a**) and TGF-β1-expressing cells (**c**) at 24, 72 and 168 h after the injury. MCP-1 and TGF-β1 (arrows) were mainly expressed in mononuclear inflammatory cells (monocytes/macrophages), fibroblasts, and endothelial cells. Both MCP-1 and TGF-β1 expressed a cytoplasm staining (**b**, **d**). Scale bar = 0.1 mm. * $p \leq 0.05$; ** $p \leq 0.01$.

Myofibroblast Analysis

Myofibroblasts were only observed 168 h after the injury (fig. 5a). After 24 and 72 h, smooth muscle cells and cells in the pericyte stained for α-SMA (data not shown). At 168 h after the injury, the wounds treated with local vitamin B complex presented a significantly higher expression of α-SMA when compared to the control ulcers ($p \leq 0.01$; fig. 5). The degree of wound contractions was correlated with an increase in contractile myofibroblasts expressing α-SMA (fig. 1, 5).

Discussion

Although many studies are available in the literature, an effective treatment of cutaneous wounds has not been clearly defined yet. According to the literature, vitamin B complex can modulate inflammation [6–8] and activate the healing process [9–20]. However, the mechanisms through which they act remain unclear. Our results are in agreement with the literature. Those ulcers treated with local vitamin B complex presented not only a significantly higher degree of contraction 168 h after the injury (fig. 1a, b), but also a larger fibroblast and vascular population at 72 and 168 h after the injury (fig. 2b, c). At 168 h after the treatment, those ulcers treated with local vitamin B complex showed a higher intensity for collagen fibril staining, suggesting a greater maturation of the granulation tissue (fig. 3c, d).

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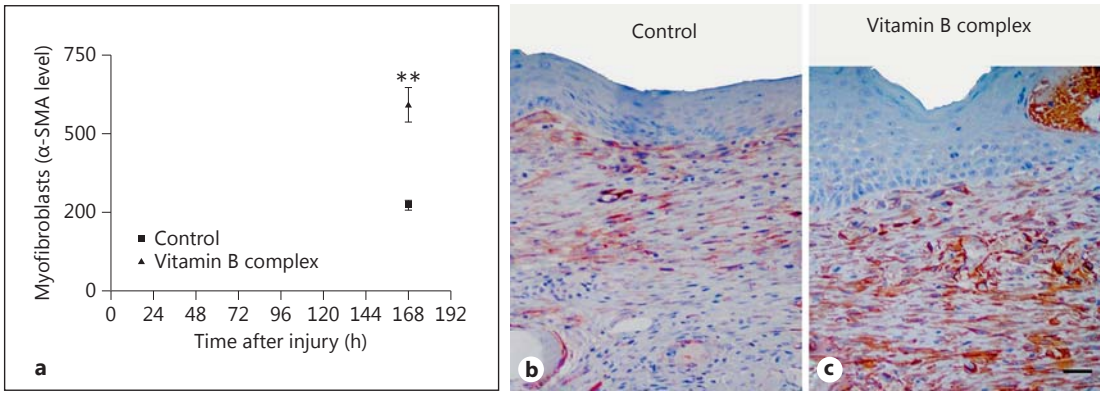

Fig. 5. a Quantification of α-SMA-expressing cells. **b**, **c** Histological features of α-SMA-expressing cells are mainly observed in myofibroblasts. Scale bar = 0.34 mm. ** $p \leq 0.01$.

Vitamin B can increase the lysyl-oxidase activity – the essential enzyme in the formation of collagen [9, 12]. The topical use of dexpanthenol, the stable alcoholic form of pantothenic acid (vitamin B5), is widely used in skin care and in the treatment of various dermatological diseases. It stimulates the regeneration and promotes the wound healing of the skin [22–24]. An upregulation of IL-6, IL-1β, CYP1B1, CXCL1, CCL8, and KAP gene expressions by vitamin B5 has been shown [24]. Biotin or vitamin B complex supplementation clinically improved the wound healing of uncomplicated sole ulcers in animals [17] or of periodontal tissue after surgery [19], respectively. Furthermore, folate supplementation stimulated DNA synthesis in the wounds of rabbits [20] as well as reduced the level of plasma homocysteine, a sulfhydryl-containing amino acid involved in the bioavailability of nitric oxide [25, 26]. According to de Lucia and Martinelli [14], fibrin is more rapidly substituted by granulation tissue in the alveoli of vitamin B6-supplemented rats when compared to a control group. Dexpanthenol also downregulated psoriasin (S100 calcium-binding protein A7A) mRNA and protein expressions [24]. Psoriasin is markedly increased in epidermal hyperproliferative disorders, in wound exudates, in parts of the epidermis surrounding acute wounds, and in the margins of nonhealing chronic leg ulcers [27].

Diverse chemokines have been suggested to play an important role in the healing process. One of these substance is MCP-1, a member of the CC family of chemokines (or β-chemokines), expressed in different kinds of cells (e.g. keratinocytes, fibroblasts, monocytes/macrophages, or endothelial cells). MCP-1, which binds to CC chemokine receptor 2, is able to stimulate the migration of monocytes/macrophages and endothelial cells; additionally, it can activate the production of the extracellular matrix [28–30]. It has also been shown that MCP-1 is related to TGF-β1 expression [31]. MCP-1 can mediate the angiogenic effect of TGF-β through the activation of vascular smooth muscle cell and mesenchymal cell migration toward endothelial cells [32, 33]. Our study showed that the MCP-1 expression was significantly higher in the experimental group than in the control group 24 h after the treatment (fig. 4a). In addition, there was a significantly larger population of mononuclear inflammatory cells (monocytes/macrophages) in the experimental group 24 and 72 h after the treatment (fig. 2a). According to the literature, the highest level of MCP-1 or its mRNA occurs during the initial phase of the healing process (after 24 h), and it diminishes during the following periods [33, 34]. Nevertheless, some researches on mice showed that the migration of monocytes/macrophages is not strictly related to the chemokine MCP-1 [30–34], but its inhibition decreased the angiogenesis and synthesis of collagen in mouse ulcers [29].

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Another essential chemokine involved in wound healing is TGF-β1. This chemokine is considered to be a member of the superfamily of polypeptides, where 3 out of 5 isoforms are produced by mammalian cells (TGF-β1, TGF-β2, and TGF-β3). This growth factor, which is produced by most cells, acts through serine/threonine kinase. TGF-β1 is able to activate the angiogenesis, fibroblastic proliferation, and production of collagenous fibers [35, 36] and macrophage migration [37, 38]. According to Mustoe et al. [37], only one application of TGF-β1 to an ear ulcer in a rabbit is sufficient to accelerate the formation of granulation tissue. In our study, TGF-β1 was significantly more expressed in ulcers topically treated with a single dose of vitamin B complex when compared to the control group 24 and 168 h after the treatment (fig. 4c).

It has also been suggested that TGF-β1 in association with another substance/event (e.g. specialized ECM proteins, like the ED-A splice variant of fibronectin, and high extracellular stress) is able to activate the formation of α-SMA-positive myofibroblasts [3, 39, 40]. Binding of TGF-β to the receptor leads to the phosphorylation of the cytoplasmic proteins called Smads, which enter the nucleus and can regulate α-SMA transcription together with other transcription factors [35, 36]. TGF-β can also accelerate the differentiation of fibrocytes into mature fibroblasts and myofibroblasts [4, 41, 42]. Different cell types such as fibrocytes, resident fibroblasts, smooth muscle cells, pericytes, and mesenchymal stromal cells have been considered as the origin of myofibroblast progenitors [2, 43]. When activated, myofibroblasts are able to contract wounded skin [40]. Furthermore, myofibroblasts can express collagen types I, III, IV, V, and VI, glycoproteins, and proteoglycans (e.g. fibronectin, laminin, or tenascin) [43]. According to our results, ulcers treated with local vitamin B complex did not only show significantly larger myofibroblast populations 168 h after the treatment (fig. 5), but also a higher contraction of the cutaneous ulcers (58.28%) when compared to the control group (32.1%; fig. 1).

It has been shown that there are two peaks of active TGF-β in the healing process. One of the peaks occurs at the initial phase of wound healing, whereas a second peak coincides with the appearance of myofibroblasts. Co-cultures of keratinocytes and fibroblasts showed high levels of active TGF-β protein after 24 and 48 h, whereas the differentiation into myofibroblasts started after 4 days or later [44]. According to Cheon et al. [45], the increase in growth factor during the initial phase is able to activate β-catenin in fibroblasts of mice in the experimental model in vitro or in vivo. β-Catenin may mediate the effect of TGF-β and regulate the wound size in cutaneous healing [46]. In addition, this protein together with nuclear protein family TCF-LEF activates the expression of genes in the late phase, e.g. metalloproteinase [45]. Our results suggest that one local application of vitamin B complex can activate the formation of granulation tissue and its maturation as well as the development of myofibroblasts and wound closure, probably through the increase of TGF-β and MCP-1 expression.

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Disclosure Statement

There are no competing conflicts of interest.

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