



# Highly Diluted Acetylcholine Promotes Wound Repair in an *In Vivo* Model

Francesca Uberti,<sup>1,\*</sup> Vera Morsanuto,<sup>1</sup> Sabrina Ghirlanda,<sup>1</sup> Sara Ruga,<sup>1</sup> Nausicaa Clemente,<sup>2</sup> Cristina Boieri,<sup>3</sup> Renzo Boldorini,<sup>3</sup> and Claudio Molinari<sup>1</sup>

<sup>1</sup>Physiology Laboratory, Department of Translational Medicine, UPO, Novara, Italy.

<sup>2</sup>Immunology Laboratory and <sup>3</sup>Unit of Pathology, Department of Health Sciences, UPO, Novara, Italy.

**Objective:** Wound healing is a dynamic, interactive, and complex process that involves a series of events, including inflammation, migration, proliferation, granulation tissue formation, and matrix remodeling. Despite the high frequency of serious slow-healing wounds, there is still no adequate therapy. The aim of this study is to evaluate a new highly diluted acetylcholine (ACh) formulation obtained through a sequential kinetic activation (SKA) method applied to a wound healing *in vivo* model to verify the hypothesis that a low dose of ACh could be a more physiological stimulus for healing, by stimulating muscarinic and nicotinic receptors and their related intracellular pathways.

**Approach:** Two different concentrations (10 fg/mL and 1 pg/mL) and two formulations (either kinetically or nonkinetically activated) of ACh were used to verify the wound healing process. Area closure, histological aspect, and nicotinic and muscarinic receptors, matrix metalloproteinase 9 (MMP-9), Nestin, and von Willebrand's factor have been assessed by Western blot or ELISA and compared to 147 ng/mL ACh, used as positive control. Moreover, the systemic effect through plasmatic radical oxygen species (ROS) production and ACh concentration has been evaluated.

**Results:** ACh SKA 1 pg/mL revealed a significant capacity to restore the integrity of tissue compared to other formulation and this effect was more evident after a single administration.

**Innovation:** Topical application on skin of ACh SKA 1 pg/mL accelerates wound closure stimulating non-neuronal cholinergic system.

**Conclusion:** Our results demonstrate for the first time the importance in an *in vivo* model of highly diluted SKA ACh during wound healing, suggesting a potential use in skin disease.

**Keywords:** wound healing, low dose, acetylcholine, sequential kinetic activation



Francesca Uberti, PhD

Submitted for publication September 19, 2017. Accepted in revised form November 15, 2017.

\*Correspondence: Physiology Laboratory, Department of Translational Medicine, UPO, Via Solaroli 17, Novara 28100, Italy (e-mail: francesca.uberti@med.uniupo.it).

## INTRODUCTION

THE SKIN CARRIES out multiple key functions, acting as an environmental protection barrier, an immune response site, and a neuroendocrine

organ.<sup>1,2</sup> Therefore each injury interrupting skin integrity has to be immediately repaired to restore its physiological functions. In this case the keratinocytes (KCs) proliferate

© Francesca Uberti *et al.*, 2018; Published by Mary Ann Liebert, Inc. This Open Access article is distributed under the terms of the Creative Commons Attribution Noncommercial License (<http://creativecommons.org/licenses/by-nc/4.0>), which permits any noncommercial use, distribution, and reproduction in any medium, provided the original authors and the source are cited.

and migrate to reepithelialize the epidermis layer and to cover the wound while underlying fibroblasts migrate and synthesize extracellular matrix proteins for connective tissue regeneration. When a tissue injury is produced in mice, fibroblasts differentiate into myofibroblasts and promote wound healing by decreasing the size of the wound.<sup>3,4</sup> Actually, a nonhealing wound requires a multifaceted approach with standard methods, including removal of necrotic tissue from wound, reduction of pressure on the wound, infection control, surgical revascularization, and limb elevation or compression. Currently, the most important bioactive approaches can be classified into three main categories, namely local delivery of growth factors,<sup>5</sup> delivery of therapeutic genes,<sup>6</sup> and delivery of stem cells.<sup>7</sup> Generally, only growth factors have been tested in large clinical trials, probably due to the safety features and logistical challenges accompanying gene or stem cell therapies. However, most growth-factor-based therapies have shown limited benefits in clinical trials for wound healing.<sup>8,9</sup> The activity of the cholinergic receptor types can regulate inter-keratinocyte communication and extracellular matrix adhesion modifying the regulation of intercellular adhesion molecules like cadherins and integrins.<sup>10</sup>

Several important functions in KC, including control of cell viability, proliferation, adhesion, migration, and differentiation, have been attributed to the non-neuronal cholinergic system, particularly muscarinic receptors.<sup>1,11,12</sup> Simultaneous stimulation of nicotinic and muscarinic receptors by acetylcholine (Ach) seems to be necessary to synchronize and balance ionic and metabolic events within the cell during epidermal turnover.<sup>13,14</sup> Some evidence has been found about the therapeutic capability of cholinomimetics or blockers in skin diseases.<sup>13,15,16</sup> For this reason, it can be hypothesized that the role of non-neuronal Ach in KC development and functions could have important clinical implications. However, as regards the therapeutic use of Ach, it must be considered that this is a highly reactive molecule and consequently its use can lead to side effects.<sup>12</sup>

A promising way could be the use of highly diluted substances as reported by several studies.<sup>17,18</sup> For example, highly diluted cytokines have been shown a significant beneficial effect in skin diseases both in patients and in cultured cells.<sup>19,20</sup> Highly diluted forms used have been obtained through a preparation method called “sequential kinetic activation” (SKA)<sup>20,21</sup>; this method was also applied to prepare a new Ach formulation used to induce healing in a wound *in vitro* model.<sup>11</sup> In this study low doses of sequentially kinetically activated Ach

seem to play an active role in an *in vitro* model of wound healing, suggesting that Ach administered in a physiological range may not only be effective but also likely to be safe. The use of SKA technique is common in different fields, including pharmaceutical technology, to obtain a high therapeutic potential at very low dosages.<sup>11</sup> Thus, the aim of this study is to evaluate the role of low doses of Ach SKA versus nonactivated diluted formulation in *in vivo* wound healing model.

## CLINICAL PROBLEM ADDRESSED

The treatments of nonhealing wounds in many cases are ineffective, leaving patients with chronic ulcers and an enhanced risk of limb amputation. In recent years, the therapeutic capability of either cholinomimetics or blockers in pemphigus and psoriatic lesions has been suggested; so the non-neuronal Ach SKA low dose could have important clinical implications for patients with several skin disorders, including nonhealing wounds and immune and inflammatory diseases. Indeed, in preclinical studies, Ach SKA low dose is safe and effective after topical application to full-thickness wounds.

## MATERIALS AND METHODS

### Preparation of Ach solutions

In this study two different concentrations of Ach (1 pg/mL and 10 fg/mL) were used. These concentrations were formulated in two distinct forms named SKA and non SKA (NO SKA), based upon the knowledge of activated blends available in literature.<sup>22,23</sup> All formulations were prepared in sterile 0.9% saline solution. The NO SKA form was a direct dilution in sterile 0.9% saline solution, whereas the SKA form was also kinetically energized by a mechanically applied force through a standardized shaking process called SKA, as previously described.<sup>11</sup> Briefly, the applied shaking procedure is characterized by vertical shaking, a 10-cm motion range, and a shaking speed corresponding to 100 oscillations in 10 s. All solutions were prepared by GUNA Laboratories (GUNA S.p.a, Milan, Italy) and donated to noiVita (noiVita s.r.l.s., Novara, Italy). The effects of SKA and NO SKA have been compared to Ach (147 ng/mL), which is known to induce KC migration.<sup>11</sup>

### Animal model

Male C57BL/6JOLA<sup>Hsd</sup> mice were purchased from Envigo<sup>+++</sup> (Bresso, Italy),  $n = 80$  of 8-week-old mice weighing 20–25 g, and were used to perform wound healing experiments. The animals had free access to food and water *ad libitum*. The experimental sub-

jects were kept in a single holding room and housed in a constant temperature of  $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , humidity of  $55\% \pm 5\%$ , and under 12-h light/12-h dark cycle. During the whole period of treatment, the mice were daily monitored to assess their health status. Blood was obtained from the tail vein after inducing anesthesia. All experimental procedures on animals were reviewed and approved by the University Committee OPBA (Organismo preposto al benessere degli animali) in accordance with local ethical standards and protocols approved by national guidelines (Approval No. 108/2016-PR).

### Experimental design

Before wounding procedure, mice were anesthetized with 4% isoflurane in an oxygen chamber 96% (1 L/min) and maintained on a heating pad, to shave the dorsal area, after cleaning skin with 10% povidone-iodine (Betadine; Sigma-Aldrich, Milan, Italy), and to make the incisional wound, following a classical protocol.<sup>24</sup> Briefly, two full-thickness wounds made by a sterile 4 mm diameter biopsy punch (Miltex, GmbH, Rietheim-Weilheim, Germany) on the middle of dorsal shaved skin were performed.

The wounds were surrounded by a 0.5 mm ring made from a thick silicone sheet with a hole in it of 5 mm. The wound was centered within the ring which was immobilized using 6-0 nylon sutures and cyanoacrylate glue to prevent wound contraction.<sup>25–27</sup> 15  $\mu\text{L}$  of each agent were added on the wounds and they were covered by a transparent occlusive water resistant patch. Finally, wounded mice were placed in individual cages and housed separately for the duration of the study. The animals were monitored daily for any sign of infection. The animals were randomized into two different protocols of treatment and sacrificed after 8 days<sup>28</sup> and they were euthanized by  $\text{CO}_2$  asphyxiation at postinjury days T0 ( $n=4$ ), 5 ( $n=12$ ), and 8 ( $n=64$ ). In protocol A ( $n=32$ ) the animals were treated at T0, checked every 24 h, and sacrificed after 8 days; in protocol B ( $n=32$ ), the animals were treated every 24 h, checked every 48 h, and sacrificed after 8 days (protocol B). In protocol A the involvement of muscarinic and nicotinic receptors ( $n=12$ ) using a specific agonist and antagonist, administered for 5 days, was tested as well; in particular bethanechol (8  $\mu\text{g}/\text{mL}$ ; Sigma-Aldrich) and scopolamine (15.21  $\mu\text{g}/\text{mL}$ ; Sigma-Aldrich) were, respectively, used as the agonist and the antagonist of muscarinic receptors. Moreover, nicotine (8.11  $\mu\text{g}/\text{mL}$ ; Sigma-Aldrich) and hexamethonium (10.12  $\mu\text{g}/\text{mL}$ ; Sigma-Aldrich), respectively, as the agonist and the antagonist of nicotinic receptor were ad-

ministered.<sup>29,30</sup> At the end of each treatment for each protocol, 4 mm diameter skin biopsy punched samples of the surrounded wound area were collected, to exclude possible circadian interferences to the outcome of the study. At the same time after treatment, blood samples were obtained and plasma was isolated to analyze radical oxygen species (ROS) production, Ach free, nestin, and matrix metalloproteinase 9 (MMP-9) concentrations in protocol A only. Two skin bioptic punches from each animal were obtained and used for the different protocols; the former was frozen in liquid nitrogen for Western blot analysis, and the latter was formalin fixed and paraffin embedded using a standard technique.

### Quantification of wound closure

The pictures of the wounds at 0, 5, and 8 days were taken using Nikon D70 camera, and the areas were quantified using ImageJ program. The closure areas were compared to day 0 (area 100% open) and to control wound (untreated). The pictures were taken at the moment of sacrifice after removing the rings. The results are expressed as percentage of the relative closure of the wounds, which was compared to the original size, using the following formula<sup>31</sup>:

$$\% \text{ wound closure} = [(WA_0 - WA)/WA_0 \times 100],$$

where

WA = wound area;

WA<sub>0</sub> = original size of the wound area.

### ROS analysis

The concentration of ROS in plasma was measured in a 96-well plate using the Antioxidant Assay Kit (Cayman Chemical, MI) following the manufacturer's instructions.<sup>32</sup> In brief, 10  $\mu\text{L}$  of metmyoglobin and 150  $\mu\text{L}$  of chromogen per well were added in plasma and standard samples (Trolox in Assay buffer from 0 to 0.33 mM) and the reactions started adding 40  $\mu\text{L}$  of Hydrogen Peroxide Working Solution to all the wells. The 96-well plate was covered, mixed for 5 min at room temperature, and the absorbance was measured using spectrophotometer (VICTOR X4 Multilabel Plate Reader) at 750 or 405 nm. The results are expressed as mean  $\pm$  standard deviation (SD) (%).

### Wound extract and Western blot analysis

At the end of each protocol the skin wounds were excised out, washed in physiological saline, weighed, cut in small pieces, and homogenized 100 mg tissue/300  $\mu\text{L}$  with cold lysis buffer (0.1 M Tris, 0.01 M NaCl, 0.025 M EDTA, 1% NP40, 1% Triton X-100;

Sigma-Aldrich, Milan, Italy) supplemented with 2 mM sodium orthovanadate, 0.1 M sodium fluoride (Sigma-Aldrich), 1:100 mix of protease inhibitors (Sigma-Aldrich), and 1:1,000 phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), using an electric potter at 1,600 rpm for 2 min. The tissue extracts were centrifuged at 13,000 rpm for 20 min at 4°C, and the protein extracts were quantified using bicinchoninic acid (BCA) assay (Protein Assay Kit; Pierce Biotechnology, Rockford, IL). Forty micrograms of each lysate were resolved on 8% SDS-PAGE gel, transferred to polyvinylidene fluoride membranes (PVDF; GE Healthcare Europe GmbH, Milan, Italy) and incubated overnight at 4°C with specific primary antibody: anti-muscarinic receptor 1 (M1, 1:250; Santa Cruz Biotechnology, Heidelberg, Germany), anti-muscarinic receptor 3 (M3, 1:250; Santa Cruz Biotechnology), anti-muscarinic receptor 5 (M5, 1:400; Santa Cruz Biotechnology), anti-nicotinic  $\alpha 7$  (1:2,000; Sigma-Aldrich), anti-MMP-9 (1:2,000; Thermo-Scientific, MA), anti-von Willebrand's factor (vWF, 1:200; Santa Cruz Biotechnology), anti-CD31 (1:200; Santa Cruz Biotechnology), anti p-44/42 MAPK (1:1,000; Euroclone, Milan, Italy), and anti-ki67 (1:200; Santa Cruz Biotechnology). Protein expression was normalized and verified through  $\beta$ -actin detection (1:5,000; Sigma-Aldrich).

### Histological analysis

Biopsy tissues were fixed in 10% buffered formalin at room temperature for 24 h, dehydrated with increasing concentrations of ethanol, embedded in paraffin, cut into 5  $\mu$ m sections, and stained with hematoxylin and eosin (Sigma-Aldrich), to assess the morphological integrity of sample tissue.<sup>33</sup> The stained sections were examined at light microscopy, and the pictures were taken at 200 $\times$  original magnification.

### Ach measurements

Choline/Ach Quantification Kit (Sigma-Aldrich) was used to determine Ach level, following the manufacturer's instructions. Briefly, the blood was centrifuged at 4,000 rpm for 10 min at 4°C to obtain the serum fraction. Twenty microliter serum sample was mixed with 30  $\mu$ L of choline assay buffer and centrifuged at 13,000 rpm for 10 min. 50  $\mu$ L of each sample were added to 50  $\mu$ L of Reaction Mix into 96-well plates on a horizontal shaker for 30 min at room temperature protected from light. The Ach concentration was determined by measuring the absorbance through a spectrometer (VICTOR X3 Multilabel Plate Reader) at 570 nm and calculated by comparing results to choline standards (0–5 nmol).

### Nestin quantification assay

Mouse NES (Nestin) ELISA Kit (Cabru, Italy) was used to quantify nestin on serum samples, following the manufacturer's instructions. Briefly, serum collected from blood samples was added in 96-well/plate and incubated for 90 min at 37°C. Then biotinylated antibody and SABC working solution were added and incubated for 60 and 30 min, respectively, at 37°C. 3,3',5,5'-tetramethylbenzidine (TMB) substrate, after incubation for 30 min at 37°C, and Stop solution were added in the plate. Nestin concentration was determined by measuring the absorbance through a spectrometer (VICTOR X3 Multilabel Plate Reader) at 450 nm and calculated by comparing results to nestin standard curve.

### MMP-9 quantification assay

MMP-9 (Mouse) ELISA Kit (DBA, Italy) was used to quantify MMP-9 on serum samples, following the manufacturer's instructions. Briefly, serum samples in 96-well/plate were incubated at 37°C for 90 min and then biotinylated antibody and ABC working solution were added and the plate incubated at 37°C for 60 and 30 min, respectively.

TMB substrate, after incubation for 15 min at 37°C, and TMB Stop solution were added. The MMP-9 concentration was determined by measuring the absorbance through a spectrometer (VICTOR X3 Multilabel Plate Reader) at 450 nm and calculated by comparing results to MMP-9 standard curve.

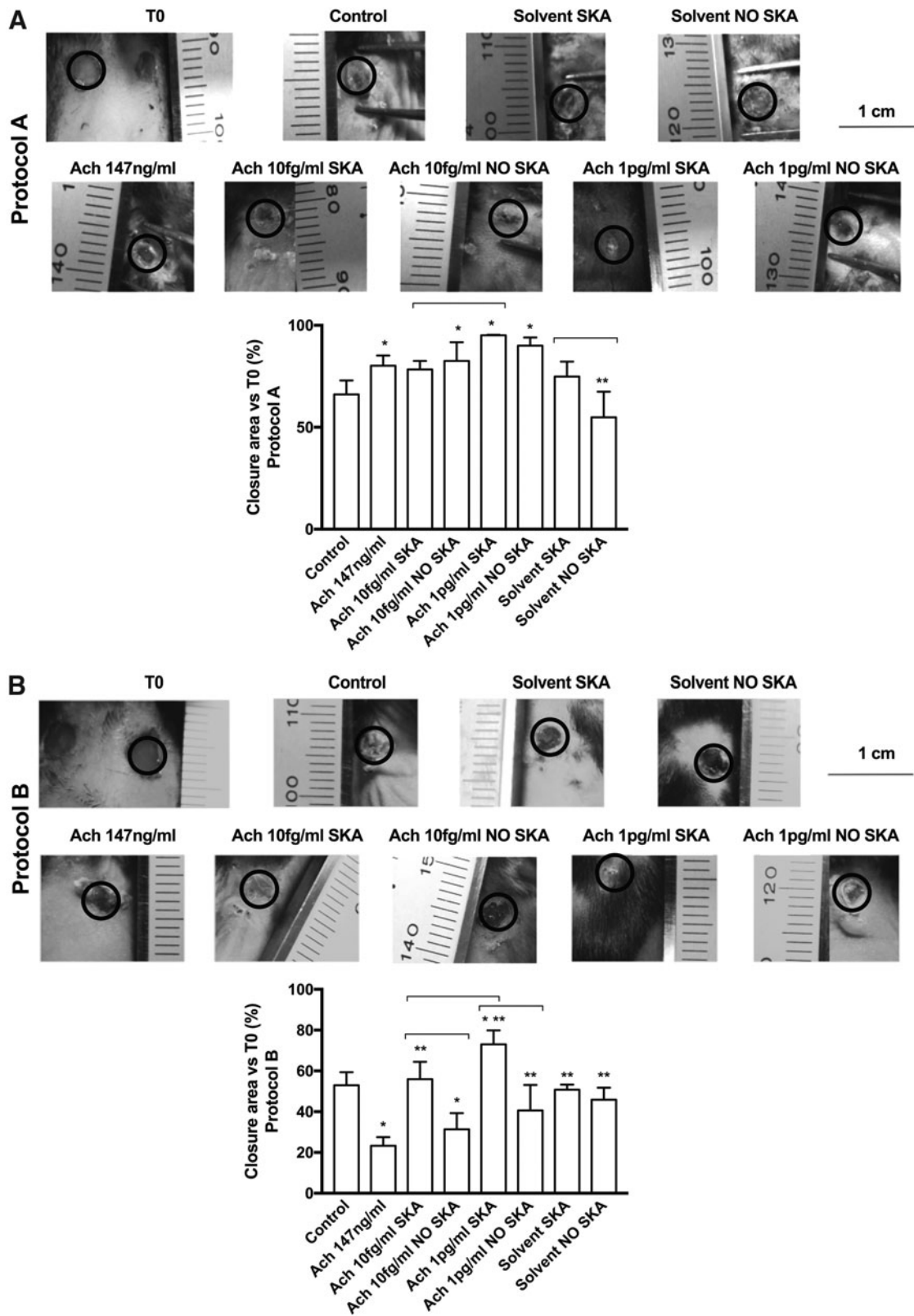
### Statistical analysis

All results were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test; data are expressed as mean  $\pm$  SD of at least three independent experiments for each experimental protocol. The percentage values were compared through Mann-Whitney *U* test. Comparisons between the two groups were performed using a two-tailed Student's *t*-test. Multiple comparisons between groups were analyzed by two-way ANOVA followed by a two-sided Dunnett *post hoc* testing. *p*-Value <0.05 was considered statistically significant.

## RESULTS

### Evaluation of wound healing area

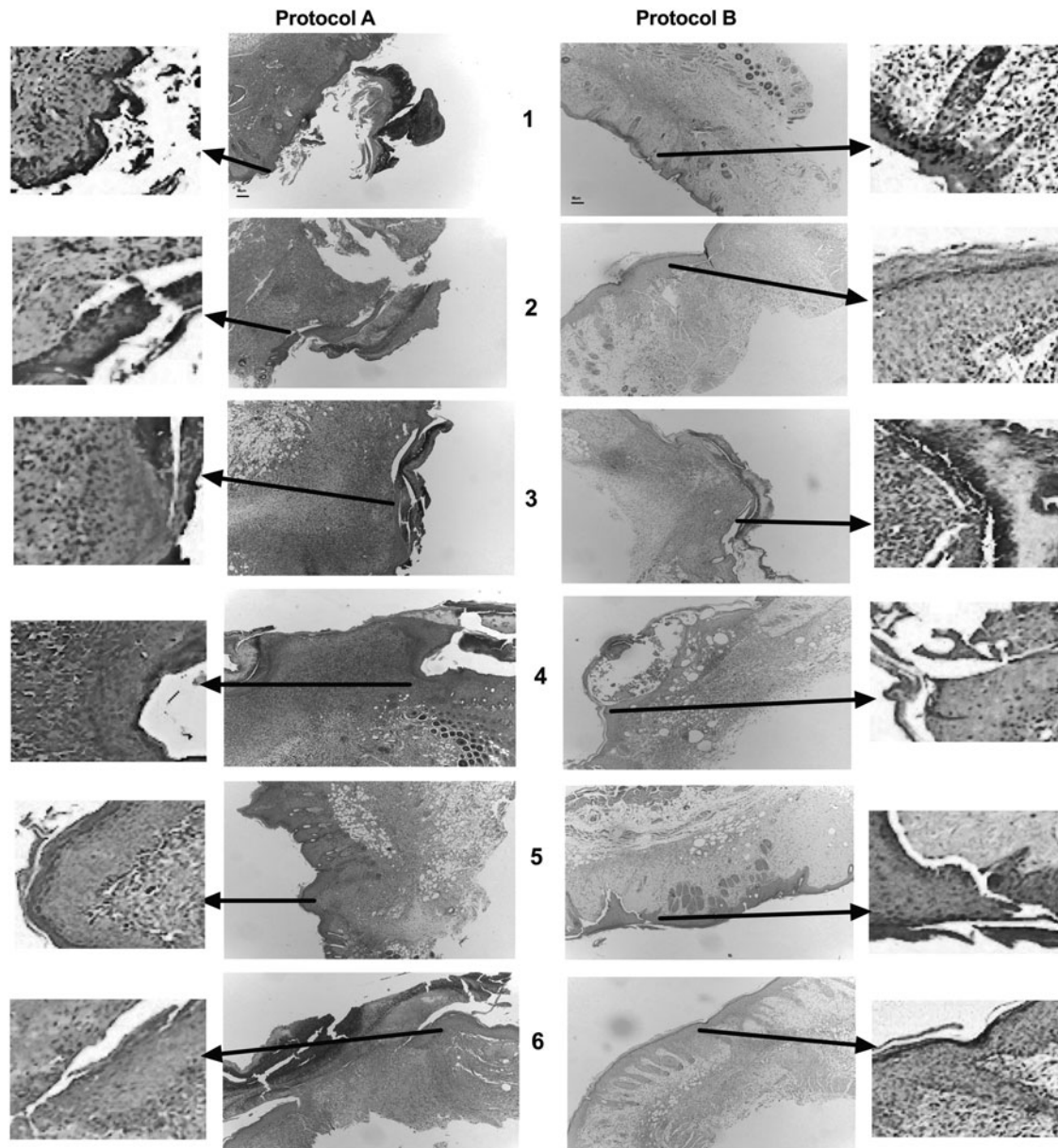
The wound healing experimental model performed in mice treated lasted 8 days using different formulations of Ach (SKA and NO SKA) in two different concentrations (10 fg/mL and 1 pg/mL). Data were compared to Ach 147 ng/mL to study the ability of Ach low doses to induce the closure of wound area. As reported in Fig. 1, there were no substantial differences between protocol A and B



**Figure 1.** Wound healing postsurgical measurements and relative wound closure graph. On both panels the quantification area of wound that may be considered as open/partially open after treatments is shown. **(A)** Data obtained in protocol A of treatment (8 days with one administration) are reported; **(B)** data obtained in presence of protocol B (8 days with one administration/day) are shown. Scale bar, 1 cm for all images and the *black circle* corresponds to the original area. \* $p < 0.05$  versus control, \*\* $p < 0.05$  versus Ach 147 ng/mL, bars ( $p < 0.05$ ) indicate significance between the activated and nonactivated forms. The results are expressed as mean  $\pm$  SD (%) compared to T0 ( $n = 32$  for each protocol). Ach, acetylcholine; NO SKA, nonsequential kinetic activation; SKA, sequential kinetic activation; SD, standard deviation.

in the eschar formation, although it appeared at a starting phase in protocol B and more mature during protocol A. In addition, in protocol A, the Ach SKA 1 pg/mL was able to induce a greater closure area than what was observed with the NO SKA form (44% and 33% vs. control, respectively) and compared to 147 ng/mL Ach (about 20%) as well. This effect was also statistically significant ( $p < 0.05$ ) in comparison with untreated wounds and to the solvent alone. In protocol B (Fig. 1B) the presence of Ach SKA 1 pg/mL was able to induce a faster closure than control (about 37%), the NO SKA 1 pg/mL (about 82%), the Ach 10 fg/mL both SKA (about

31%) and NO SKA (about 128%), respectively. However, Ach SKA 1 pg/mL was able to exert a greater effect in protocol A than in B (30% protocol A vs. protocol B). Indeed, Ach SKA 1 pg/mL appears to induce an almost or fully complete healing demonstrating the importance of dilution and SKA. In both protocols Ach 147 ng/mL was not able to restore the structural integrity of the damaged area. These findings have been confirmed by morphological analysis (Fig. 2), in which Ach SKA 1 pg/mL seems to be a better choice. In protocol A the mice treated with Ach SKA 1 pg/mL presented a complete epithelialization (shown within circle) of the wound



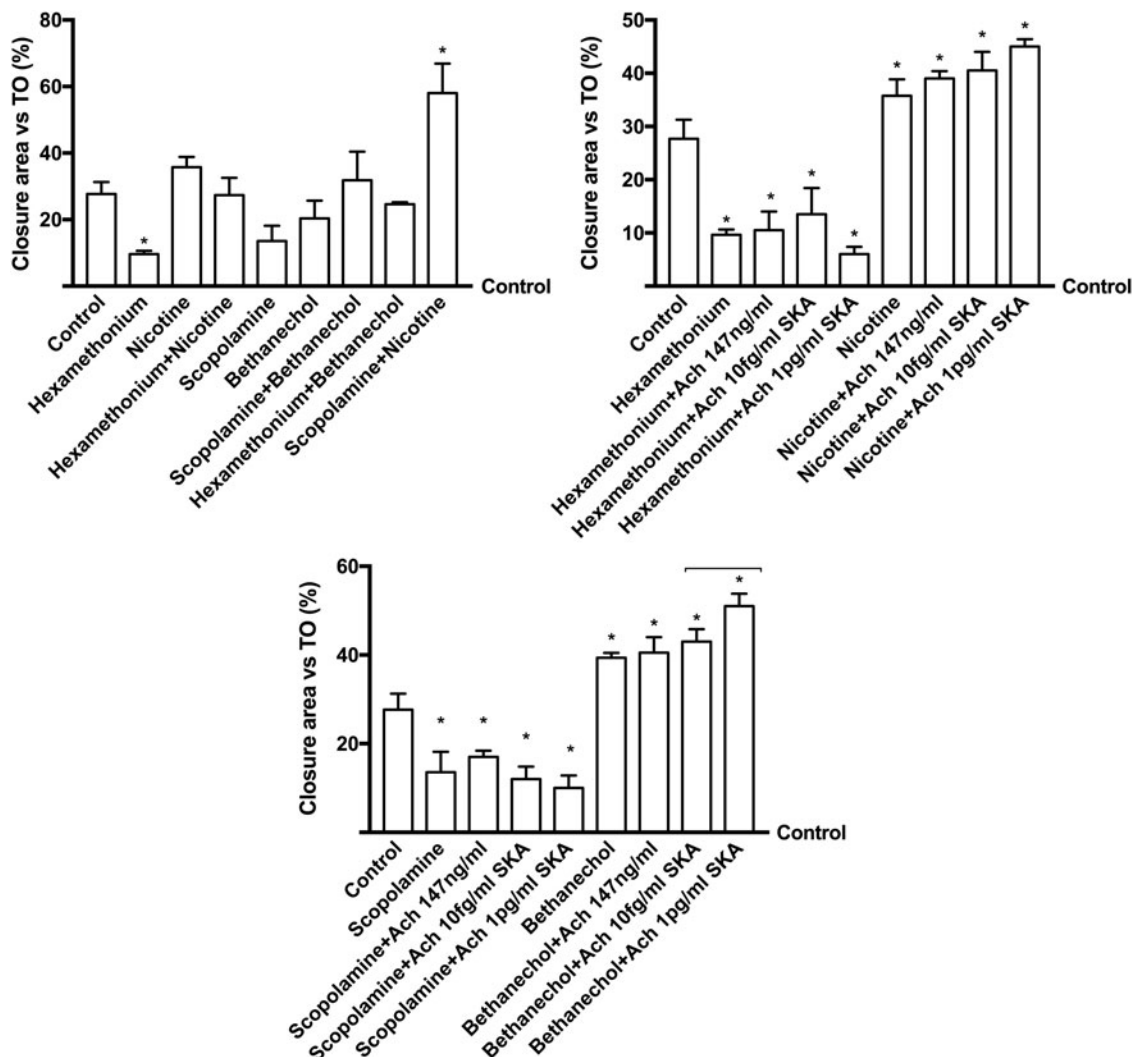
**Figure 2.** Hematoxylin and eosin stained and sectioned mouse wound tissue. Representative microscopy pictures of wound healing with each treatment with protocol A ( $n=18$ ) and protocol B ( $n=18$ ) are reported at original magnification of 200 $\times$  and are the representative examples. The scale reported is to be considered valid for all treatments. 1= control; 2=Ach 147 ng/mL; 3=Ach 10 fg/mL SKA; 4=Ach 10 fg/mL NO SKA; 5=Ach 1 pg/mL SKA; 6=Ach 1 pg/mL NO SKA.

compared to control, to the same NO SKA form, to the Ach 10 fg/mL both SKA and NO SKA, respectively. In particular, epithelialization appeared without any ulcer, with the presence of inactive granulation tissue, with collagen remodeling and angiogenesis; for this reason it is possible to envisage a *restitutio ad integrum*. The same concentration in NO SKA showed poor capacity to heal wound, limited to an initial state, and appearing ulcerated. Ach SKA 10 fg/mL was different from Ach SKA 1 pg/mL because the granulation tissue was still very active with a superficial ulceration; the same NO SKA concentration induced a condition similar to SKA 10 fg/mL, but the ulceration was more extensive. In contrast, in all treatments of protocol B (Fig. 2), the granulation tissue seemed to be still very active, and the healing was at an early stage. In particular, Ach SKA 1 pg/mL showed a quite evident capacity to repair wound; reepithelialization was

almost complete, but in the underlying layer fibroblasts, sclerosis, and granulation tissue were still present. Finally, another important finding was obtained from Ach 147 ng/mL administration in both protocols; in particular only in protocol A an initial remodeling compared to control was observed, confirming the importance of Ach dosage.

#### Involvement of MMP-9, vWF, nestin, and muscarinic and nicotinic receptors

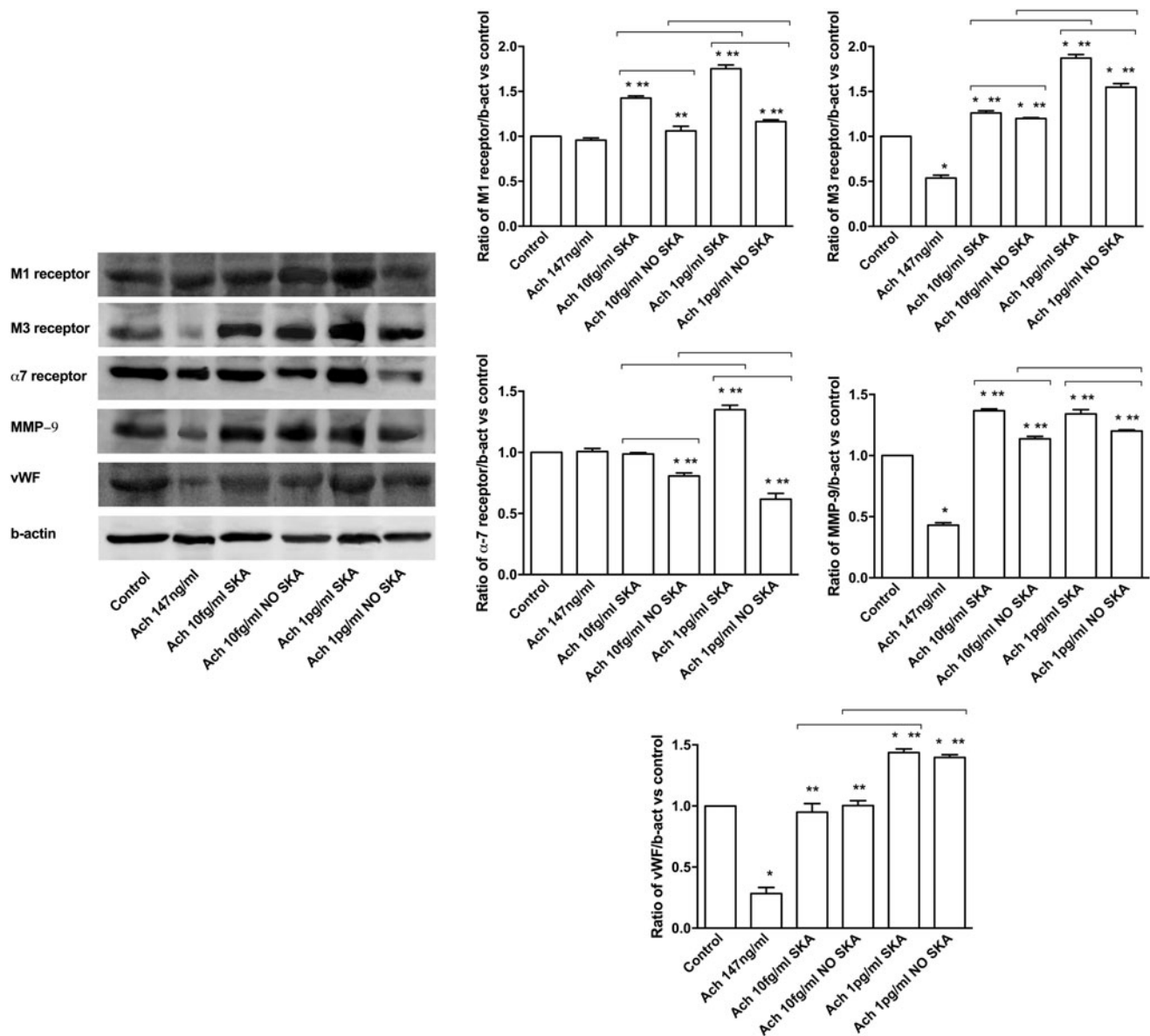
To determine whether there was an involvement of nicotinic and muscarinic receptors in healing, specific agonists and antagonists were tested in protocol A. As reported in Fig. 3 bethanechol (8  $\mu$ g/mL; M1 and M5 agonist) and nicotine (8.11  $\mu$ g/mL;  $\alpha$ 7 agonist) did not induce any significant changes compared to control ( $p > 0.05$ ) on the damaged area. Scopolamine (15.21  $\mu$ g/mL; M1 antagonist) and hexamethonium (10.12  $\mu$ g/mL;  $\alpha$ 3 $\beta$ 2 antagonist)



**Figure 3.** Representative wound closure graph of anticholinergic drugs performed in protocol A. The results are expressed as mean  $\pm$  SD ( $n=12$ ). \* $p < 0.05$  versus control, bars ( $p < 0.05$ ) indicate significance between agonistic and antagonist agents.

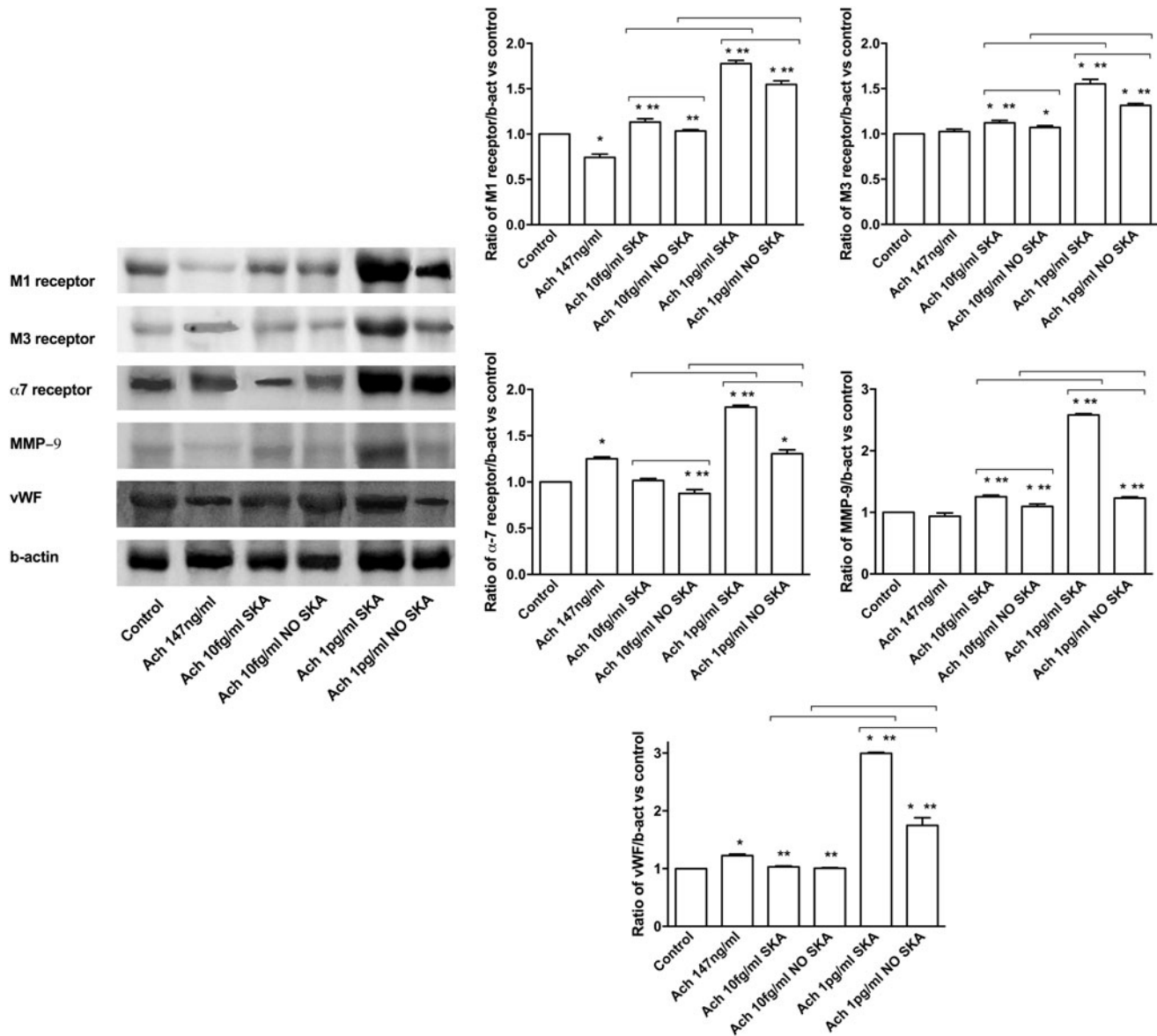
prevented any physiological healing compared to control (50% and 64%, respectively). Using both agonist and antagonist combined together, a significant reduction in healing was observed ( $p < 0.05$ ) compared to control, confirming the active role of M1, M3, and  $\alpha 7$ . For this purpose, in the subsequent experiments the effects of different formulations of Ach on these receptors were also investigated. The involvement of these receptors on Ach mechanisms was also confirmed by the analysis of experiments performed in presence of Ach 147 ng/mL and Ach SKA (10 fg/mL and 1 pg/mL).

As shown in Figs. 4 and 5, the importance of the involvement of the M1, M3, and  $\alpha 7$  receptors in both protocols was clarified by Western blot and densitometric analysis, and the best results were obtained with protocol A. In particular in protocol A (Fig. 4), in presence of Ach SKA 1 pg/mL the M1 and M3 expressions were more evident compared to control (73% and 55%, respectively), to Ach SKA 10 fg/mL, and compared to both NO SKA concentrations ( $p < 0.05$ ). In addition Ach SKA 1 pg/mL was able to enhance the expression of  $\alpha 7$  compared to control (about 81%), confirming the link among M1, M3, and  $\alpha 7$  receptors in healing. On the



**Figure 4.** Western blot and densitometric analysis of muscarinic receptors (M1 and M3), nicotinic ( $\alpha 7$ ) receptor, MMP-9, and vWF markers in protocol A. Protein extracts were analyzed by immunoblotting with specific antibodies against the indicated proteins and normalized through  $\beta$ -actin detection. Data are expressed as mean  $\pm$  SD (%) of four biological replicates normalized to control values. The pictures are examples for each treatment of the experimental protocol. \* $p < 0.05$  versus control, \*\* $p < 0.05$  versus Ach 147 ng/mL, bars ( $p < 0.05$ ) indicate significance between the activated forms, the nonactivated forms, and between the two different forms. MMP-9, matrix metalloproteinase 9; vWF, von Willebrand's factor.



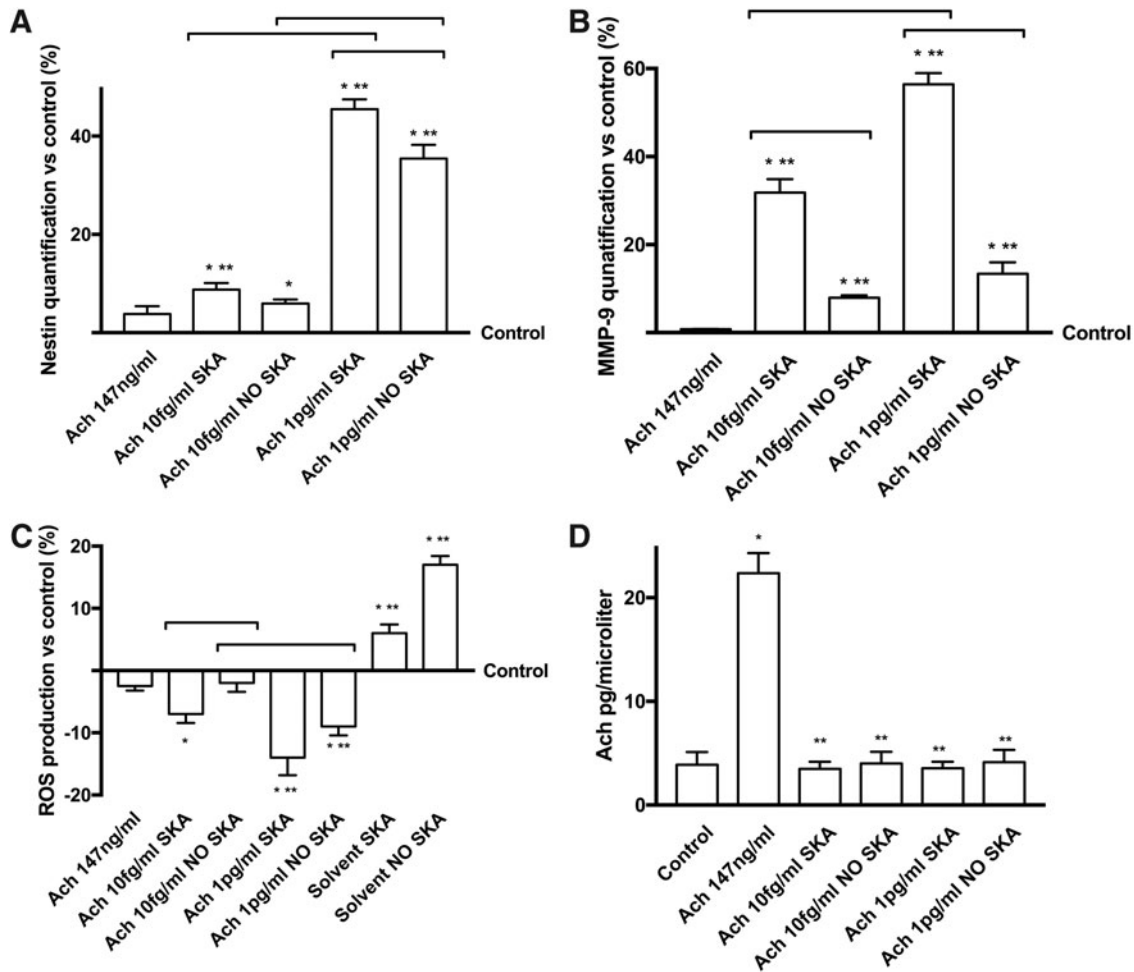


**Figure 5.** Western blot and densitometric analysis of muscarinic receptors (M1 and M3), nicotinic ( $\alpha 7$ ) receptor, MMP-9, and vWF markers in protocol B. Protein extracts were analyzed by immunoblotting with specific antibodies against the indicated proteins and normalized through  $\beta$ -actin detection. Data are expressed as mean  $\pm$  SD (%) of four biological replicates normalized to control values. The pictures are an example of each treatment of the experimental protocol. \* $p < 0.05$  versus control, \*\* $p < 0.05$  versus Ach 147 ng/mL, bars ( $p < 0.05$ ) indicate significance between the activated forms, the nonactivated forms, and between the two different forms.

contrary, in protocol B (Fig. 5) Ach SKA 10 fg/mL and 1 pg/mL were able to induce M1 and M3 expressions compared to control (10 fg/mL: 43% M1, 26% M3; 1 pg/mL: 75% M1, 87% M3), and Ach SKA 1 pg/mL enhanced the expression of  $\alpha 7$  receptor of about 35% compared to control. In both protocols Ach 10 fg/mL and 1 pg/mL NO SKA was effective only on the M1 and M3 receptors. Finally, Ach 147 ng/mL was not able to induce expression of receptors in a sufficient manner to generate healing.

MMP-9 and vWF were also analyzed in both protocols; as reported in Figs. 4 and 5, Ach SKA was able to act early on healing, in particular, on for-

mation of cell matrix and on adhesion in lesion area. In particular, in protocol A both Ach 10 fg/mL and 1 pg/mL SKA forms ( $p < 0.05$ ) appeared to be more effective than control to act on the collagen remodeling (eschar formation, late stage of healing), using the MMP-9 marker, and compared to Ach NO SKA; in protocol B both concentrations of Ach SKA caused an increase of MMP-9 compared to control (36% 10 fg/mL; 34% 1 pg/mL) and to the same NO SKA forms (10 fg/mL 13%; 1 pg/mL 20%). Finally, as regards the terminal stages of healing, Ach SKA was able to complete the healing process acting on angiogenesis, as assessed by the



**Figure 6.** Analysis of systemic nestin, MMP-9, ROS, and Ach quantification level with protocol A. **(A)** Nestin activity is expressed as mean  $\pm$  SD (%) of eight biological replicates normalized to control values and indicated as a percentage respect to the control. **(B)** MMP-9 activity is expressed as mean  $\pm$  SD (%) of eight biological replicates normalized to control values and indicated as a percentage with respect to the control. **(C)** ROS production is expressed as mean  $\pm$  SD (%) of eight biological replicates normalized to control values and indicated as percentage with respect to the control. **(D)** Ach quantification is expressed as mean  $\pm$  SD (%) of eight biological replicates normalized to control values. \* $p < 0.05$  versus control, \*\* $p < 0.05$  versus Ach 147 ng/mL, bars ( $p < 0.05$ ) indicate significance between the activated forms, the nonactivated forms, and between the two different forms. ROS, radical oxygen species.

study on marker vWF (involved in new blood vessels). In particular, in protocols both A and B, only Ach SKA 1 pg/mL was able to enhance vWF production ( $p < 0.05$ ) compared to control. In addition, as reported in Fig. 6, the activities of nestin (Fig. 6A) and MMP-9 (Fig. 6B) activities were analyzed on plasma samples in protocol A since this appeared as the most effective protocol. Ach SKA forms showed a greater increase in MMP-9 compared to control ( $p < 0.05$ ), and, in particular, a significant increase was observed in presence of Ach SKA 1 pg/mL compared to the Ach SKA 10 fg/mL ( $p < 0.05$ ) and Ach 147 ng/mL ( $p < 0.05$ ), following previous data about the remodeling of the matrix. In addition, the activity of nestin, the neoangiogenesis marker, confirmed the hypothesis about the vascularization; indeed Ach SKA 1 pg/mL induced a significant

increased activity of nestin compared to control ( $p < 0.05$ ) and Ach 147 ng/mL ( $p < 0.05$ ) and 10 fg/mL SKA ( $p < 0.05$ ). These data support the results observed with morphological analysis. These results showed an active role of Ach SKA 1 pg/mL in migration, healing, and angiogenesis and proved that the single treatment was the most effective. Therefore, the final analysis will be performed using protocol A only.

#### ROS analysis and Ach quantification

The plasmatic production of ROS during healing is a physiological process. However, their levels should be kept low, otherwise beneficial effects would turn into cytotoxic. As reported in Fig. 6A, in presence of Ach SKA 1 pg/mL the level of ROS was less than control ( $p < 0.05$ ), as well as it was

compared to other Ach forms ( $p < 0.05$ ), confirming its active role. Ach 147 ng/mL did not induce an excessive release of ROS, thus providing a positive condition to begin the healing process. Both Ach SKA and NO SKA 1 pg/mL kept levels of ROS lower than Ach 147 ng/mL ( $p < 0.05$ ). In addition to exclude a systemic effect of Ach, the analysis on its plasma concentration during protocol A was also performed (Fig. 6B). Both Ach SKA and NO SKA did not increase the plasma Ach concentration, and the differences among results obtained with different forms and concentrations were not significant. On the contrary Ach 147 ng/mL significantly increased the circulating amount of Ach and it could cause a systemic effect (about 470% compared to control). The treatments with both concentrations of Ach SKA and NO SKA were not able to act systemically, since plasma concentration remained unvaried.

## DISCUSSION

Poor wound healing after trauma, surgery, acute illness, or chronic diseases affects millions of people worldwide each year, and current therapies have limited effectiveness.<sup>34</sup> The aim of this study was to examine the possible use of low-dose Ach SKA as an innovative therapy for the stimulation of non-neuronal cholinergic system in healing. The effectiveness of low doses of cytokines is well known by a study on asthmatic mice,<sup>17</sup> and in many clinical studies, for example, they were used as supportive care or as pain relief in cancer patients,<sup>35,36</sup> or to reduce symptoms in patients with psoriasis or vitiligo.<sup>19,21,37,38</sup> Further data have also shown the improvements in patients with cutaneous mucus diseases treated with cholinergic agents.<sup>39</sup> In this context it was important to demonstrate in an *in vivo* model the ability of low-dose Ach SKA during healing, suggesting a potential use in dermatology. Ach is produced in all types of living cells and it is greatly abundant on surface epithelium,<sup>15</sup> in particular in the human skin (*i.e.*, Ach 1,000 pmol/g) where it is synthesized by choline acetyltransferase and hydrolyzed by acetylcholinesterase.<sup>40</sup> A single KC synthesizes an average of  $2 \times 10^{-17}$  mol of Ach and gives  $7 \times 10^{-19}$  mol/min.<sup>13</sup> This finding accounts for the dosage of Ach SKA used in this study and allows us to consider the concentrations as physiological (10 fg/mL and 1 pg/mL). Reepithelialization is an Ach-dependent process mediated by muscarinic and nicotinic receptors. Both *in vitro*<sup>11</sup> and *in vivo* studies showed the ability of Ach SKA 1 pg/mL and Ach SKA 10 fg/mL to act on both muscarinic (particularly M1 and M3) and nicotinic receptors ( $\alpha 7$ ). Two different protocols of

treatment were used to assess the best posology in an *in vivo* model of wound. In particular, protocol A was characterized by a single stimulation, whereas protocol B consisted of repeated administrations every 24 h.

Data observed showed that protocol A had greater effectiveness than protocol B and that Ach SKA 1 pg/mL was able to repair lesion more rapidly than Ach SKA 10 fg/mL. We investigated the closure area, the intracellular mechanism activated, the ROS produced, and the eventual systemic effect of low dose Ach. An important element was the role of muscarinic and nicotinic receptors. Data obtained in the *in vivo* model on muscarinic/nicotinic receptors showed that ultra-diluted Ach acts through the same mechanisms activated by Ach 147 ng/mL, which supports the hypothesis of beneficial effects exerted by the low dose in the absence of adverse side effects (such as oxidative stress) as reported by low levels of ROS concentration observed in plasma samples.

Hence the modulation of receptor activity can be hypothesized; in both protocols M1 and  $\alpha 7$  receptors acted in a decoupled way (M1 increasing, while  $\alpha 7$  decreasing) and M3 and  $\alpha 7$  receptors acted in a coupled way (increasing together). This can be seen in the treatment with SKA 1 pg/mL in protocol A. Moreover, the treatment with Ach 147 ng/mL in the two protocols demonstrated the dose limit in wound; in protocol B Ach 147 ng/mL was not able to start healing (migration), but in protocol A healing began and continued in the later stages of migration, as shown by molecular markers (vWF and MMP-9 and nestin). The SKA forms (1 pg/mL and 10 fg/mL) showed a greater ability to trigger healing compared to Ach 147 ng/mL and these effects were more evident in the presence of a single treatment (protocol A).

The evaluation of vWF, MMP-9, and nestin was important to understand whether the healing process has fully started leading to a *restitutio ad integrum* or it was just at the initial steps. Data from this study demonstrate for the first time that Ach SKA 1 pg/mL was able to activate all stages of healing effectively when administered only once (protocol A) compared to the same concentration of NO SKA, to Ach SKA 10 fg/mL, and to Ach 147 ng/mL, respectively. In addition, Ach SKA 1 pg/mL was able to reduce and maintain the level of ROS at a basal concentration and did not induce any significant changes on Ach concentration in blood, confirming that the beneficial effects observed had no negative systemic effects. These data demonstrate for the first time an *in vivo* model that low doses of Ach could be important for the development of new

therapeutic strategies for wound healing. In addition these findings suggest that the cholinergic agents could have a large potential use in dermatology. The observation of an increased effect induced by Ach SKA has been demonstrated for the first time *in vivo* model within the non-neuronal cholinergic system.

### KEY FINDINGS

- Topical application of low-dose Ach SKA heals full-thickness cutaneous wounds stimulating non-neuronal cholinergic system.
- Low-dose Ach SKA promotes angiogenesis and migration of KC, through the balance between nicotinic and muscarinic receptors.
- Preclinical data confirmed that Ach SKA at low doses is safe, effective, and represents an alternative solution for the treatment of wounds.

### INNOVATION

Ach SKA low doses for topical treatment of both nonhealing/healing or acute/chronic cutaneous wounds may be an innovative therapeutic strategy. Ach SKA can be safe for topical administration without either systemic or adverse effects. A single administration of Ach SKA could easily and rapidly restore epithelium, thus it may represent a convenient therapeutic strategy for patients with relatively low costs.

### ACKNOWLEDGMENTS AND FUNDING SOURCES

The authors thank Ms. Mariangela P. Fortunato for her help with language revision. GUNA S.p.a (Vincenzo Miranda) contributed donating solutions. Work was supported by private funding of Physiology laboratory and by Fondazione Giovanni Goria and Fondazione CRT “2015.”

### AUTHOR DISCLOSURE AND GHOSTWRITING

No ghostwriting was involved, and there are no disclosures related to any author.

### ABOUT THE AUTHORS

**Francesca Uberti, PhD**, is a post doc and Professor of General Pathology and has supervised funded research on use of Ach SKA for the treatment of wounds. She is a co-founder and CEO & CTO of start-up spin-off “noiVita srls” at University of Eastern Piedmont. **Claudio Molinari, MD**, works at the Physiology Laboratory. **Vera Morsanuto, MS**, is a PhD student at Physiology Laboratory. **Sabrina Ghirlanda, MS**, and **Sara Ruga, BS**, are the Master students in Biotechnology at Physiology Laboratory. **Nausicaa Clemente, PhD**, is a post-doc in Immunology Laboratory. **Cristina Boieri, MS**, is a member of the unit of Pathology directed by Prof. **Renzo Boldorini, MD**.

### REFERENCES

- Kurzen H, Wessler I, Kirkpatrick CJ, Kawashima K, Grando SA. The non-neuronal cholinergic system of human skin. *Horm Metab Res* 2007;39:125–135.
- Slominski AT, Zmijewski MA, Skobowiat C, Zbytek B, Slominski RM, Steketeer JD. Sensing the environment: regulation of local and global homeostasis by the skin's neuroendocrine system. *Adv Anat Embryol Cell Biol* 2012;212:v, vii, 1–115.
- Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature* 2008;453:314–321.
- Jain N, Kalailingam P, Tan KW, Tan HB, Sng MK, Chan JS, et al. Conditional knockout of N-WASP in mouse fibroblast caused keratinocyte hyper proliferation and enhanced wound closure. *Sci Rep* 2016;6:38109.
- Goldman R. Growth factors and chronic wound healing: past, present, and future. *Adv Skin Wound Care* 2004;17:24–35.
- Branski LK, Pereira CT, Herndon DN, Jeschke MG. Gene therapy in wound healing: present status and future directions. *Gene Ther* 2007;14:1–10.
- Zahorec P, Koller J, Danisovic L, Bohac M. Mesenchymal stem cells for chronic wounds therapy. *Cell Tissue Bank* 2015;16:19–26.
- Buchberger B, Follmann M, Freyer D, Huppertz H, Ehm A, Wasem J. The evidence for the use of growth factors and active skin substitutes for the treatment of non-infected diabetic foot ulcers (DFU): a health technology assessment (HTA). *Exp Clin Endocrinol Diabetes* 2011;119:472–479.
- Später T, Frueh FS, Metzger W, Menger MD, Laschke MW. In vivo biocompatibility, vascularization, and incorporation of Integra® dermal regenerative template and flowable wound matrix. *J Biomed Mater Res B Appl Biomater* 2016;106:52–60.
- Fania L, Zampetti A, Guerriero G, Feliciani C. Alteration of cholinergic system in keratinocytes cells produces acantholysis: a possible use of cholinergic drugs in pemphigus vulgaris. *Antiinflamm Anti-allergy Agents Med Chem* 2012;11:238–242.
- Uberti F, Bardelli C, Morsanuto V, Ghirlanda S, Cochis A, Molinari C. Stimulation of the nonneuronal cholinergic system by highly diluted acetylcholine in keratinocytes. *Cells Tissues Organs* 2016;203:215–230.
- Beck B, Zholos A, Sydorenko V, Roudbaraki M, Lehen'kyi V, Bordat P, et al. TRPC7 is a receptor-operated DAG-activated channel in human keratinocytes. *J Invest Dermatol* 2006;126:1982–1993.
- Grando SA, Kist DA, Qi M, Dahl MV. Human keratinocytes synthesize, secrete, and degrade acetylcholine. *J Invest Dermatol* 1993;101:32–36.
- Grando SA, Pittelkow MR, Schallreuter KU. Adrenergic and cholinergic control in the biology of epidermis: physiological and clinical significance. *J Invest Dermatol* 2006;126:1948–1965.
- Grando SA. Cholinergic control of epidermal cohesion. *Exp Dermatol* 2006;15:265–282.
- Grando SA. Pemphigus autoimmunity: hypotheses and realities. *Autoimmunity* 2011;45:7–35.
- Gariboldi S, Palazzo M, Zanobbio L, Dusio GF, Mauro V, Solimene U, et al. Low dose oral administration of cytokines for treatment of allergic asthma. *Pulm Pharmacol Ther* 2009;22:497–510.

18. D'Amico L, Ruffini E, Ferracini R, Roato I. Low dose of IL-12 stimulates T cell response in cultures of PBMCs derived from non-small cell lung cancer patients. *J Cancer Ther* 2012; 3:337–342.
19. Barygina V, Becatti M, Lotti T, Moretti S, Taddei N, Fiorillo C. Treatment with low-dose cytokines reduces oxidative-mediated injury in perilesional keratinocytes from vitiligo skin. *J Dermatol Sci* 2015;79:163–170.
20. Radice E, Miranda V, Bellone G. Low-doses of sequential-kinetic-activated interferon- $\gamma$  enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer: a preliminary study. *Int Immunopharmacol* 2014;19:66–73.
21. Roberti ML, Ricottini L, Capponi A, Sclauzero E, Vicenti P, Fiorentini E, et al. Immunomodulating treatment with low dose interleukin-4, interleukin-10 and interleukin-11 in psoriasis vulgaris. *J Biol Regul Homeost Agents* 2014;28:133–139.
22. Brod SA, Khan M. Oral administration of IFN-alpha is superior to subcutaneous administration of IFN-alpha in the suppression of chronic relapsing experimental autoimmune encephalomyelitis. *J Autoimmun* 1996;9:11–20.
23. Avvakumov EG, Chizhevskaya SV, Stoyanov ES, Povetkina MV, Chekmarev AM, Shafirov VL, et al. Influence of the nature of components in mechanically activated mixture of zirconium and silicon oxides on solid-phase synthesis of zircon. *Russ J Appl Chem* 1999;72:1498–1503.
24. Dunn L, Prosser HC, Tan JT, Vanags LZ, Ng MK, Bursill CA. Murine model of wound healing. *J Vis Exp* 2013;28:e50265.
25. Das S, Singh G, Majid M, Sherman MB, Mukhopadhyay S, Wright CS, et al. Syndesome therapeutics for enhancing diabetic wound healing. *Adv Healthc Mater* 2016;5:2248–2260.
26. Jain AK, Tewari-Singh N, Inturi S, Orlicky DJ, White CW, Agarwal R. Histopathological and immunohistochemical evaluation of nitrogen mustard-induced cutaneous effects in SKH-1 hairless and C57BL/6 mice. *Exp Toxicol Pathol* 2014;66:129–138.
27. Fairweather M, Heit YI, Buie J, Rosenberg LM, Briggs A. Celecoxib inhibits early cutaneous wound healing. *J Surg Res* 2015;194:717–724.
28. Buszewska-Forajta M, Siluk D, Dagher-Wojtkowiak E, Sejda A, Staškowiak D, Biernat W, et al. Studies of the effect of grasshopper abdominal secretion on wound healing with the use of murine model. *J Ethnopharmacol* 2015;176:413–423.
29. Miyamoto T, Nojima H, Kuraishi Y. Intradermal cholinergic agonists induce itch-associated response via M3 muscarinic acetylcholine receptors in mice. *Jpn J Pharmacol* 2002;88:351–354.
30. Khurana S, Shah N, Cheng K, Shiu B, Samimi R, Belo A, et al. Scopolamine treatment and muscarinic receptor subtype-3 gene ablation augment azoxymethane-induced murine liver injury. *J Pharmacol Exp Ther* 2010;333:639–649.
31. Pinto NC, Cassini-Vieira P, Souza-Fagundes EM, Barcelos LS, Castañón MC, Scio E. *Pereskia aculeata* Miller leaves accelerate excisional wound healing in mice. *J Ethnopharmacol* 2016;194:131–136.
32. Kampa M, Nistikaki A, Tsaousis V, Maliraki N, Notas G, Castanas E. A new automated method for the determination of the total antioxidant capacity (TAC) of human plasma, based on the crocin bleaching assay. *BMC Clin Pathol* 2002;2:3–18.
33. Lattuada D, Uberti F, Colciaghi B, Morsanuto V, Maldì E, Squarzanti DF, et al. Fimbrial cells exposure to catalytic iron mimics carcinogenic changes. *Int J Gynecol Cancer* 2015;25:389–398.
34. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. *Sci Transl Med* 2014;6:265sr6.
35. Rajendran ES. Homeopathy as a supportive therapy in cancer. *Homeopathy* 2004;93:99–102.
36. Sunila ES, Kuttan R, Preethi KC, Kuttan G. Dynamized preparations in cell culture. *Evid Based Complement Alternat Med* 2009;6:257–263.
37. Witt CM, Lüdtke R, Willich SN. Homeopathic treatment of patients with psoriasis—a prospective observational study with 2 years follow-up. *J Eur Acad Dermatol Venereol* 2009;23:538–543.
38. Kho MM, Bouvy AP, Cadogan M, Kraaijeveld R, Baan CC, Weimar W. The effect of low and ultra-low dosages Thymoglobulin on peripheral T, B and NK cells in kidney transplant recipients. *Transpl Immunol* 2012;26:186–190.
39. Kuwahara RT, Skinner RB, Rosenberg EW. Nicotine gum for oral lichen planus. *J Dermatol* 2000;27:755.
40. Klapproth H, Reinheimer T, Metzén J, Münch M, Bittinger F, Kirkpatrick CJ, et al. Non-neuronal acetylcholine, a signalling molecule synthesized by surface cells of rat and man. *Naunyn Schmiedebergs Arch Pharmacol* 1997;355:515–523.

#### Abbreviations and Acronyms

$\alpha 7$	= nicotinic receptor
Ach	= acetylcholine
ANOVA	= analysis of variance
KC	= keratinocyte
M1	= muscarinic type 1 receptor
M3	= muscarinic type 3 receptor
M5	= muscarinic type 5 receptor
MMP-9	= matrix metalloproteinase 9
NO SKA	= nonsequential kinetic activation
ROS	= radical oxygen species
SD	= standard deviation
SKA	= sequential kinetic activation
TMB	= 3,3',5,5'-tetramethylbenzidine
vWF	= von Willebrand's factor
WA	= wound area
WA <sub>0</sub>	= original size of the wound area